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14. ABSTRACT

This proposal has evaluated the activity of the Pim protein kinase. This enzyme has been shown to function with the c-Myc protein and enhance tumor growth. It is possible that Pim enhanced the growth of tumor cells by stimulating growth and then also decreasing proteins that would normally inhibit the growth of cells. The goals of this research were to study the ability of Pim to regulate hepatocyte growth factor/ cMet signaling, examine the regulation of the p27 cell cycle inhibitory protein by Pim, and investigate the interaction of the Pim and AKT pathways in driving Pim mediated growth. The results of these studies have led to an understanding of Pim-1 mechanism of action and three publications in peer-reviewed journals. In this report, these findings are detailed with a focus on the ability of Pim to upregulate c-Met, downregulate p27, and enhance protein synthesis in the face of AKT inhibition.

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INTRODUCTION (Subject, purpose, and scope)

The overexpression of the serine/threonine Pim protein kinase in normal or cancerous prostate cells stimulates the growth of these cells. The reason for this increase in growth is not fully understood and was the subject of this grant. Pim is over expressed in human prostate cancer, and its level may parallel the onset of metastatic disease. In animal models of prostate cancer, the expression of the c-Myc protein is associated with increased Pim protein levels in these tumor cells. Thus, data from tissue culture, human and animal prostate cancer implicates the Pim protein in prostate tumorigenesis.

In this application our preliminary data suggested that the overexpression of Pim in prostate cancer 1) regulates the Met tyrosine kinase receptor and HGF levels to control the growth migration and transformation of prostate cancer cells; 2) modulates the level of the p27 protein causing changes in cell growth; and 3) enhances the growth of prostate cancer based on AKT and Pim to play a role in stimulating prostate cancer growth. The ability of Pim to drive down the level of p27 suggests that the levels of this protein could be important in affecting tumor growth. We have hypothesized that this decrease will coordinate with elevated c-Myc in tumor cells to modulate the growth Potentially, both AKT and Pim could collaborate to stimulate and enhance tumor occurrence.

To explore these potential possibilities our laboratory has developed novel benzylidene thiazolidine-2,4 diones, (D5, SMI-4a) that inhibit the activity of Pim kinase. We have shown in preliminary data that these agents reverse Pim activity and allow the level of p27 to rise. The purpose of this grant was to explore the mechanism of action of Pim in prostate cancer and to develop novel data that would enhance our understanding of this disease and allow for the further development of targeted therapy.

BODY
Task 1: Clarify the biochemical mechanism by which Pim acts as an "internal promoter" of prostate cell growth and migration.

Subtask 1a: Determine if Pim-1 overexpression enhances the secretion of HGF/SF stimulating growth migration and transformation of prostate cells.

Using prostate cancer cells overexpressing Pim-1 data accumulated in this grant **(3)** demonstrates that these cells express increased amounts of cell surface c-Met receptor. These Pim-1 containing cells demonstrate increased motility in a scratch assay and increased motility in the Boyden chamber assay. Injecting prostate cancer cells either expressing Pim or not in the tail vein of mice demonstrates that the Pim expressing cells form increased metastatic sites, suggesting that increases in c-Met may enhance Pim dependent metastatic behavior. To understand how Pim may be controlling c-Met we have cloned the 5' upstream region, a potential ribosome entry site or IRES into a plasmid. The overexpression of Pim stimulates this IRES suggesting that Pim is controlling translation of this mRNA to increase c-Met levels. We find that Pim is enhancing translation in a 5'cap independent fashion. Experiments are ongoing to examine this mechanism and determine the exact proteins regulated by Pim within this translation complex. Additional experiments show that inhibition of AKT kinase increases Pim and enhances c-Met on the cell membrane. These increases in c-Met can then drive growth through the ERK pathway. We have shown **(3)** that a combination of AKT and

Pim inhibitors can block the growth of prostate cells and tumors, suggesting that control of prostate cancer might arise from the combination of the two agents.

Subtask 1b: Elucidate the mechanism by which Pim-1 overexpression markedly reduces p27 protein levels and increases cancer cell proliferation.
The data obtained (1) demonstrates the novel observation that the Pim-1 protein kinase through a dual mechanism can regulate the levels and hence the activity of Skp2. Pim-1 is capable of binding and phosphorylating Skp2 and stabilizing protein levels, but does not affect the interaction of Skp2 with the E2 ligase Ubc3. Conversely, both siRNA and small molecule Pim-1 inhibitors decrease Skp2 levels and phosphorylation. Skp2 is phosphorylated by CDK2 at Ser64 and Ser72 and by Akt1 at Ser72 to stabilize this protein . Pim-1 appears capable of phosphorylating Skp2 at these two sites, as well as a unique site in the C terminus, Thr417 that is highly conserved throughout the animal kingdom, including humans and mice. Phosphorylation of this site is required for maximal Skp2 activity and stabilization of Skp2 protein levels in vivo. In the prostate cancer cell line PC3 that contains an activated Akt, a small molecule Pim inhibitor SMI-4a but not wortmannin or the Akt inhibitor GSK690693 decreased the levels of Skp2. LY294002, which inhibits both Akt and Pim, displayed an effect similar to that of SMI-4a, suggesting that in this cell line the Pims are essential for the regulation of Skp2 levels. Unlike Akt, Pim-1 kinase did not appear to regulate Skp2 subcellular localization. The Pim kinases share multiple similarities with AKT. It is possible that the relative abundance of each of these Skp2-phosphorylating kinases may decide which is essential to the control of Skp2 levels.

It is quite surprising that our Skp2 S72A mutant did not lose p27 degradation activity compared with the wild type Skp2 because two previous studies demonstrated that this very same Skp2 mutant completely lost ubiquitin ligase activity. However, another two recent reports confirmed our finding. The half-life of this mutant was indeed shorter than that of wild-type Skp2, consistent with previous reports. The degradation of Skp2 is regulated by APC/CCdh1 complex that preferentially associates with non-phospho-Ser64 form of Skp2. Pim-1 kinase activity does not affect the binding of Cdh1 to total Skp2, but does impair the interaction between Cdh1 and CDC27. Interaction with CDC27/APC3 protein allows Cdh1 to activate the APC/C. Although Cdh1 is inhibited by both the Emi-1 protein and multiple phosphorylations initiated in part by cyclin A-CDK2 and cyclin B1-CDK1, it has been proposed that an additional kinase may play a role. Here, we demonstrate that Cdh1 is a phosphorylation target of Pim-1 and the knockdown of Pim-1 with siRNA reduces Cdh1 phosphorylation during S, G2, and M phases, demonstrating the critical involvement of Pim-1 in tightly controlled Cdh1 phosphorylation during the cell cycle. It remains unknown whether Pim-1 and CDKs share some phosphorylation sites on Cdh1. Further studies are required to determine the precise Pim-1 sites and how these two different types of kinases cooperate to control Cdh1 activity. Interestingly, the levels of Pim-1 protein are correlated with Cdh1 phosphorylation during cell cycle progression as high Pim-1 expression and high Cdh1 phosphorylation were seen during S, G2, and M phases, and the opposite occurred during the G1 phase. Given the role of Cdh1 in regulating mitosis, this may explain why Pim-1 is not only required for Skp2 to signal S phase entry, but also plays a critical role in G2 /M phase regulation. Consistent with this hypothesis, mouse embryo fibroblasts that are knocked out for all three Pim kinase isoforms display increased number of cells in the G2 /M phase of the cell cycle. These observations are in concert with previous discoveries suggesting that Pim-1 functions in mitosis. Therefore, the Pim-1 kinase regulates Skp2 levels through the Pim-1 kinase activity, reduces APC/CCdh1 E3 ligase

activity, and thus protects Skp2 from degradation. The Pim-1 protein kinase is abnormally elevated in human cancers, regulated by growth factors, and collaborates with other oncogenes to induce cell transformation. The ability of this enzyme to modulate the activity of both the SCFSkp2 and APC/Cdh1 and thus control p27 levels is likely to be essential to the biological activities of this protein kinase.

Task 2: Evaluate the ability of novel Pim inhibitors to block the transformation of prostate cells and the progression of tumor growth.

Subtask 2a: Use animal models to test the activity of Pim inhibitors in regulating prostate transformation.

As a preliminary test of whether combined inhibition of AKT and Pim kinases might provide synergistic antitumor efficacy, we tested (3) the effects of the inhibitors on the proliferation of PC3-LN4 cells in vitro. Treatment of PC3-LN4 cells with the Pim inhibitor SMI-4a in combination with the AKT inhibitor GSK690693 resulted in a synergistic enhancement of the inhibition of proliferation as shown by combination index of less than 0.5, and a markedly greater reduction in both the numbers and the size of colonies seen in a soft-agar colony formation assay. GSK690693 and SMI-4a blocked the proliferation of DU145 in a similar fashion. To test the activity of these agents in vivo, PC3-LN4 cells were injected into mice and treated with GSK690693 alone, SMI-4a alone, or both drugs in combination on a daily basis for 21 days starting at 15 days after tumor implantation. When used alone, treatment of these drugs caused a modest inhibition of tumor growth, whereas the combined treatment resulted in a markedly greater inhibition of tumor growth. Immunoblot analysis of lysates of tumors harvested at the termination of the experiment on day 36 had up- regulated the levels of MET, EPHA2, and HER3 protein in mice treated with GSK690693 as compared with the tumors from mice treated with vehicle. Interestingly, the levels of Pim-1 were increased in the combined therapy, and could suggest an in vivo interaction between these agents cannot be ruled out. This upregulation of the RTKs was significantly reduced in the tumors from mice treated with a combination of GSK690693 and SMI-4a.

Subtask 2b: Examine whether Pim-1 enhances the prostate transforming activity of Akt.

The results of these experiments (3) provide insights into the mechanisms underlying the compensatory interplay between AKT and Pim-1 in the regulation of prostate cancer cell behavior influenced by the expression of RTKs. They suggest a model in which reduction in AKT activity is associated with an increase in the levels of Pim-1 protein kinase that occurs through a transcriptional mechanism. This increase in Pim-1 kinase is associated, in turn, with promotion of the expression of RTKs through a cap-independent mechanism. Down regulation of Pim-1 blocks the feedback elevation in RTKs associated with inhibition of AKT. Likewise inhibitors of Pim synergize with small-molecule AKT inhibitors to block the growth of prostate cancer cells. The control of Pim-1 protein levels is complex and has been shown to involve the ubiquitin proteasome pathway and translational mechanisms. We show that inhibition of AKT can increase the levels of Pim-1 through a transcriptional mechanism; however, it is possible that additional alternative mechanisms could also play a role in increasing Pim-1 protein levels. The induction of Pim-1 by AKT inhibition coincides with suppression of total protein synthesis and is not inhibited by further treatment with mTORC inhibitors, suggesting that Pim-1 protein levels could also be regulated in a cap-independent manner. The Pim-1-UTR may contain an IRES that could also be regulated by specific cellular growth conditions, although the existence of this IRES is controversial.

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We have demonstrated that Pim controls the levels of protein synthesis (2). It has been shown previously that inhibition of AKT regulates the transcription of RTKs by modulating the activity of Foxo transcription factors; however, in the same study no change was seen in the level of HER2, RET, or MET mRNAs, suggesting that the levels of specific RTKs might be controlled by other mechanisms. Cap-dependent translation plays a role in both PI3K/AKT and Pim-2 enhancement of the synthesis of specific proteins. It should be noted, however, that molecules that blockedmTORC1activity could not inhibit the Pim-2 protein kinase and an agent that blocked eIF4A function, which is known to take part in IRES mediated translation, was required. In addition, small-molecule mTORC inhibitors can decrease the translation of many mRNAs, for example, 50- terminal oligopyrimidine tracts mRNAs, while increasing the level of translation of RTKs, again suggesting that these RTK mRNAs may be translated in a cap-independent fashion. Moreover, further inhibition of cap-dependent translation with the mTORC1/2 inhibitors, PP242 and AZD8055, had no effect on the ability of SK690693 or Pim-1 to induce RTKs, suggesting that in the experimental conditions used in these studies, the mechanism by which this agent controls RTK levels is not cap dependent (3).

Our results are consistent with the hypothesis put forward that inhibition of PI3K/mTOR could lead to enhanced cap-independent translation. Cloning of the Met -UTR into a dicistronic luciferase vector showed that it can function as an IRES element, although weakly in comparison with viral sequences, and its activity is enhanced by GSK690693 and Pim-1 overexpression. Further supporting evidence of the ability of GSK690693 and Pim-1 to regulate the activity of the IRES is the observation that the IGF-IR IRES is stimulated by these agents and that Pim-1 knockdown decreases the activity of this element. Our data further suggest that Pim-1 may be essential for full IRES activity of additional viral and cellular IRES elements, including HCV, CrPV, HIF1a, and Myc, suggesting a general role of Pim in the control of cap-independent translation. It has been suggested previously that because they are both survival kinases, AKT and Pim protein kinases could be important pharmacologic targets to inhibit tumor growth. Our experiments show a high degree of synergism between small molecule inhibitors of AKT and Pim in their ability to kill.

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KEY RESEARCH ACCOMPLISHMENTS

a. Expression of Pim-1 controls the levels of p27Kip1 by modulating the phosphorylation of Skp-2 and CDC27 proteins.

b. Overexpression of Pim-1 in the mouse and human primary prostate epithelial cells appears to increase the activation of the c-Met receptor.

c. In primary prostate cells, intracellular overexpression of Pim-1 controls the migration of prostate epithelial cells in the scratch assay and through the collagen matrix.

d. Pim-1 over expression in prostate cancer cells, as well as cervical and brain cancer increases the levels of the c-Met and EGFR receptors.

e. Tissue microarray staining of human prostate cancer samples demonstrates that the levels of c-Met and Pim-1 correlate.

f. The addition to prostate cancer of pan-AKT inhibitors elevates the level of the c-Met and EGF receptors in prostate cancer tissue culture.

g. Inhibition of Pim-1 either with siRNAs or with Pim inhibitors decreases c-Met levels.

h. The combination of small molecule AKT and Pim inhibitors is synergistic in killing prostate cancer.

i. Inhibition of AKT in prostate cancer cells elevates the levels of both Pim-1, but not Pim-2 or Pim-3, and cell surface receptor tyrosine kinases (RTKs).

j. Inhibition of Pim-1 with inhibitor, SMI-4a, or decreasing the level of Pim-1 protein inhibited the ability of AKT inhibitors to induce RTKs.

k. AKT inhibition leads to the transcriptional up regulation of Pim-1.

l. Pim-1 or small molecule AKT inhibitors are able to increase the levels of RTKs by a cap-independent mechanism.

m. The combination of a Pim-1 and AKT inhibitor is synergistic in killing prostate cancer cells in tissue culture and in an animal model.

REPORTABLE OUTCOMES

Abstracts
Kraft AS, Cen B, Zemskova M, Beharry Z, Smith C. Potent protein kinase inhibitors block Pim kinase mediated increase in prostate epithelial cell migration, regulation of p27 protein half-life and secretion of hepatocyte growth factor. AACR Molecular Targets and Cancer Therapeutics Conference, 2009.

Bo Cen, Sandeep Mahajan, and Andrew S. Kraft Overcoming Resistance to Inhibitors of the AKT Protein Kinases by Targeting the Pim Protein Kinase Pathway. Advances in Prostate Cancer Research February 6-9, 2012 Abstract C12, Page 133

Papers
Cen B, Sandeep M, Zemskova M, Beharry Z, Lin YW, Cramer SD, Lilly M, and **Kraft AS.** Regulation of SKP2 Levels by the PIM-1 Protein Kinase. J Biol Chem 285 (38): 29128-29137, 2010. PMID 20663873, PMCID: PMC2937943

Beharry Z, Mahajan S, Zemskova M, Lin Y-W, Tholanikunnel B, Xia Z, Smith CD, and **Kraft AS.** The Pim protein kinases regulate energy metabolism and cell growth. Proceedings of the National Academy of Sciences. 108:528-533, 2011, PMID: 21187426, PMCID: PMC3021022

Cen B, Mahajan S, Wang W, and **Kraft AS.** Elevation of Receptor Tyrosine Kinases by Small Molecule AKT Inhibitors in Prostate Cancer Is Mediated by Pim-1. Cancer Research 73: 1–10. 2013

CONCLUSIONS

The serine threonine Pim protein kinases are overexpressed in prostate cancers and promote cell growth and survival. The PI3K/AKT pathway is activated in over 60% of human prostate cancers, suggesting that compounds that inhibit this pathway may be useful for therapy. Therefore the mechanism by which Pim might interacts with PI3K/AKT to drive tumorigenesis is important. These experiments have demonstrated that Pim is able to (1) increase the level of growth factor receptors (RTKs), e.g. c-Met, (2) decrease the level of p27, a protein that would inhibit cell growth, and (3) overcome the inhibition of AKT to stimulate RTK elevation. We demonstrate that inhibition of AKT in prostate cancer cell lines not only induces the expression of multiple RTKs, but increases the protein levels of serine threonine protein kinase Pim-1. Pim-1 activity is identified as essential in the feedback regulation of RTK levels by AKT inhibition. Knockdown of Pim-1 expression or inhibition of Pim-1 activity with small molecules abrogates the induction of RTKs and overexpression of Pim-1 increases RTK levels. Experiments using dual luciferase vectors demonstrate that Pim-1 controls expression of c-Met and other RTKs at the translational level by modulating IRES activity in a cap-independent manner. Both tissue culture and animal experiments demonstrate that the combination of AKT and Pim inhibitors provides synergistic inhibition of tumor growth. Our results demonstrate that Pim-1 is a novel mediator of resistance to AKT inhibition, and that targeting Pim kinases significantly improves the efficacy of AKT inhibitors in anticancer therapy. This combination therapy could be brought into the clinic as a therapy for prostate cancer.

APPENDIX

1- Cen B, Sandeep M, Zemskova M, Beharry Z, Lin YW, Cramer SD, Lilly M, and **Kraft AS**. Regulation of SKP2 Levels by the PIM-1 Protein Kinase. J Biol Chem 285 (38): 29128-29137, 2010. PMID 20663873, PMCID: PMC2937943

2- Beharry Z, Mahajan S, Zemskova M, Lin Y-W, Tholanikunnel B, Xia Z, Smith CD, and **Kraft AS**. The Pim protein kinases regulate energy metabolism and cell growth. Proceedings of the National Academy of Sciences. 108:528-533, 2011, PMID: 21187426, PMCID: PMC3021022

3- Cen B, Mahajan S, Wang W, and **Kraft AS**. Elevation of Receptor Tyrosine Kinases by Small Molecule AKT Inhibitors in Prostate Cancer Is Mediated by Pim-1. Cancer Research 73: 1–10. 2013

Regulation of Skp2 Levels by the Pim-1 Protein Kinase*⃞ˢ

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The Pim-1 protein kinase plays an important role in regulating both cell growth and survival and enhancing transformation by multiple oncogenes. The ability of Pim-1 to regulate cell growth is mediated, in part, by the capacity of this protein kinase to control the levels of the p27, a protein that is a critical regulator of cyclin-dependent kinases that mediate cell cycle progression. To understand how Pim-1 is capable of regulating p27 protein levels, we focused our attention on the SCF^{Skp2} ubiquitin ligase complex that controls the rate of degradation of this protein. We found that expression of Pim-1 increases the level of Skp2 through direct binding and phosphorylation of multiple sites on this protein. Along with known Skp2 phosphorylation sites including Ser^{64} and Ser^{72}, we have identified Thr^{417} as a unique Pim-1 phosphorylation target. Phosphorylation of Thr^{417} controls the stability of Skp2 and its ability to degrade p27. Additionally, we found that Pim-1 regulates the anaphase-promoting complex or cyclosome (APC/C complex) that mediates the ubiquitination of Skp2. Pim-1 phosphorylates Cdh1 and impairs binding of this protein to another APC/C complex member, CDC27. These modifications inhibit Skp2 from degradation. Marked increases in Skp2 caused by these mechanisms lower cellular p27 levels. Consistent with these observations, we show that Pim-1 is able to cooperate with Skp2 to signal S phase entry. Our data reveal a novel Pim-1 kinase-dependent signaling pathway that plays a crucial role in cell cycle regulation.

The Pim family of serine/threonine kinases regulates the growth and survival of cells and plays a role in enhancing the transformed phenotype driven by oncogenes, including Myc and Akt (1–3). As the Pim kinases are elevated in human tumors, including prostate, leukemia, and pancreatic cancer, and appear to be useful in distinguishing benign from malignant tumors (4), it has been suggested that they play a role in the growth or progression of these malignancies (5, 6). In prostate cancer, decreased Pim-1 expression correlated significantly with measures of poor outcome and was found to be associated with a higher cumulative rate of prostate-specific antigen fail-

ure and a strong predictor of prostate-specific antigen recurrence (4). Based on crystal structural analysis (7–11), the Pim family of kinases appears to be constitutively active and not regulated by a kinase cascade. To explain the ability of the Pim protein kinases to regulate growth and survival, research has initially focused on the ability of these protein kinases to regulate CDC25A and CDC25C, p21^{Waf1}, and the C-TAK1^2 protein kinase (12–14). Recently, Pim-1 has been shown to increase the cyclin-dependent kinase-2 activity, by decreasing the levels of p27^{Kip1} (p27) protein (15). Similarly, we have demonstrated that small molecule inhibitors of Pim-1 both translocate the p27 protein to the nucleus and markedly increase its levels (16, 17), suggesting that inhibiting Pim-1 activity may regulate the cell cycle by controlling p27 levels and localization.

The SCF^{Skp2} ubiquitin ligase (Skp1/cullin/F-box protein) targets cell cycle negative regulators p27, p21^{Waf1}, and p130 (18) to the proteasome for degradation and controls progression through the cell cycle. A key protein in this complex Skp2 binds phosphorylated p27 and is responsible for its destruction. The fact that increased Skp2 expression is frequently found in many cancers (19, 20) and Skp2 overexpression can drive cell transformation suggests the importance of the levels of this protein in regulating cell growth (19, 21, 22). The amount of the Skp2 protein in cells is tightly regulated by multiple pathways, including phosphorylation and proteasome degradation. The anaphase-promoting complex or cyclosome (APC/C) is active from mitosis to late G$_1$ (23, 24) and functions as the E3 ligase for this protein when activated by Cdh1 (25, 26). Phosphorylation of Skp2 by CDK2 (27) and Akt1 (28, 29) on Ser^{64} and Ser^{72} protects it from degradation by the APC/C^{Cdh1} complex and elevates the levels of this protein. However, the role of Skp2 Ser^{72} phosphorylation is under debate as contradictory findings have been reported (30, 31). Further studies are required to elucidate fully the mechanisms by which cells regulate Skp2 levels.

Here, we demonstrate that Pim-1 kinase activity stabilizes and increases the levels of Skp2 protein, thus decreasing p27 levels and promoting cell cycle progression. Pim-1 both binds Skp2 and phosphorylates it on Ser^{64} and Ser^{72}, but also on a novel site, Thr^{417}. Furthermore, Pim-1 phosphorylates Cdh1,

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ˢ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5 and additional references.
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² The abbreviations used are: C-TAK1, Cdc25C-associated kinase 1; APC/C, anaphase-promoting complex or cyclosome; HGF, hepatocyte growth factor; SCF, Skp1/cullin/F-box protein.

impairing its association with CDC27 and inhibiting APC/C activity, thus protecting Skp2 from degradation.

EXPERIMENTAL PROCEDURES

Antibodies, Drugs, and Reagents—Anti-Pim-1 (19F7) antibody was produced and purified in this laboratory. Anti-cyclin E (HE12), anti-Met (25H2), anti-phospho-Met (D26), Myc tag (71D10), anti-AKT, anti-phospho-AKT (S473), and anti-polo-like kinase-1 antibodies were purchased from Cell Signaling Technology. Anti-p27 (C19), anti-CDC27 (AF3.1), and anti-cyclin B1 (H-433) were from Santa Cruz Biotechnology. Anti-β-actin (AC-15), anti-FLAG M2, anti-HA (HA-7), and anti-β-tubulin (TUB 2.1) antibodies were from Sigma. Anti-Skp2 and anti-Cks1 antibodies were from Invitrogen/Zymed Laboratories Inc.. Anti-His tag antibody was from Qiagen. Anti-Cdh1(DH01) antibody was from Abcam. Anti-lamin B antibody was from Calbiochem.

Roscovitine and reagents for *in vitro* ubiquitination assay were from Biomol. Cycloheximide, MG132, LY294002, wortmannin, nocodazole, and thymidine were from Sigma. GSK690693 was provided by Glaxo Smith Kline.

Recombinant human HGF was from Antigenix America. Active GST-tagged Pim-1 was from SignalChem. Active His-tagged human Pim-1 was purified from *Escherichia coli* using a Calbiochem nickel-nitrilotriacetic acid column. GST and GST-Skp2 proteins were purified from *E. coli* using glutathione-Sepharose 4B resin (GE Healthcare).

Plasmids—A Pim-1 siRNA plasmid and the control plasmid were described previously (32). pGIPZ Pim-1 shRNA constructs were from Open Biosystems.

pCMV-Skp2 plasmid expressing FLAG-tagged Skp2 was kindly provided by Dr. Liang Zhu (33). Site-directed mutants were prepared using PCR based on this plasmid. HA-Cdh1 and HA-Cdc20 plasmids were described elsewhere (34). The Ubc3 and ubiquitin plasmids have been previously described (35). The HA-Pim-1 and FLAG-Pim-1 constructs were generated by subcloning murine Pim-1 cDNA into pcDNA3 vector, and the K67M (HA-tagged) mutant was constructed using PCR. An N-terminally truncated mutant (NT81) of Pim-1 was described previously (36). Lentiviral expression constructs pLEX-Pim-1 and pLEX-Skp2 was obtained by subcloning human Pim-1 and Skp2 cDNAs into pLEX vector (Open Biosystems). A human Pim-1 construct, pcDNA3-Pim-1, was described elsewhere (32).

Cell Culture, Transfections, Transductions, and Cell Synchronization—Cell lines were grown in RPMI 1640 medium (PC3) or DMEM (HeLa, HEK293T, Rat1, and mouse embryonic fibroblasts). The triple knock-out mouse of the Pim-1, -2, -3 genes used to isolate mouse embryonic fibroblasts were described previously (17). Mouse prostate epithelial cells were isolated as described (37). HEK293T cells were transfected by the calcium phosphate method, and HeLa cells were transfected with Lipofectamine 2000 reagent. Lentiviruses were produced and transduced into Rat1 cells using kits from Open Biosystems.

For synchronization experiments, HeLa cells were treated with 2 mM thymidine for 18 h, washed, and released into fresh medium for 9 h. Then, a second thymidine treatment was

applied to yield cells at the G_1/S transition. Mitotic HeLa cells were obtained by treating HeLa cells with 2 mM thymidine for 24 h, washing, and releasing into fresh medium for 3 h. The cells were then treated with 100 ng/ml nocodazole for 12 h.

Ubiquitination Assays—*In vitro* p27 ubiquitination assays were performed essentially as described (38). In brief, the SCF^{Skp2} complex was expressed and purified from insect cells (39) and mixed with *in vitro*-translated ^{35}S-labeled p27 that had previously been incubated with cyclin E/Cdk2 along with methylated ubiquitin and ubiquitin aldehyde for 60 min at 30 °C. The reaction was stopped with 2× SDS sample buffer and run on polyacrylamide gels. *In vivo* ubiquitination assays were performed as described (40). HEK293T cells were transfected with the indicated plasmids for 24 h, treated with 10 μM MG132 for 6 h, and lysed in denaturing buffer (6 M guanidine-HCl, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 10 mM imidazole). The cell extracts were then incubated with nickel beads for 3 h, washed, and subjected to immunoblot analysis.

In Vitro and in Vivo Phosphorylation Assay—FLAG-Skp2 or its mutants were immunoprecipitated with anti-FLAG antibody from HEK293T cells. Immune complexes were washed three times in radioimmune precipitation assay lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS), then washed twice in 1× kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na$_3$VO$_4$, 10 mM MgCl$_2$, 2 μM unlabeled ATP) and incubated with 0.5 μg of recombinant active Pim-1 kinase and 2 μCi of [γ-^{32}P]ATP in 3 μl of total reaction buffer for 30 min at 30 °C. Phosphorylation of Cdh1 or Cdc20 was detected using *in vitro* translated proteins produced by TNT Coupled Reticulcyte Lysate System (Promega). Reactions were stopped by washing twice in kinase buffer and boiling in 2× SDS loading buffer. Proteins were resolved by 9% SDS-PAGE, and ^{32}P incorporation was detected by autoradiography. For *in vivo* labeling experiments, HeLa cells were transfected with the indicated plasmids for 24 h, and the medium was changed to phosphate-free DMEM with 0.5% dialyzed FBS containing 200 μCi ml^{-1} ortho-^{32}PO$_4$ for 4 h. Cells were lysed by radioimmune precipitation assay buffer for immunoprecipitation, and the immune complexes were subjected to 9% SDS-PAGE followed by autoradiography analysis.

Flow Cytometry—Cell cycle distribution was monitored by FACS analysis of ethanol-fixed, propidium iodide-stained cells on a Becton Dickinson FACSCalibur Analytical Flow Cytometer.

BrdU Incorporation Assay—Rat1 cells were seeded in 96-well plates (3000 cells/well) and maintained as described in the figure legends. An ELISA BrdU kit (Roche Applied Science) was used to assay the cell cycle. Absorbance at 370 nm (reference wavelength 492 nm) was measured using a Molecular Devices microplate reader.

Densitometry Analysis—Densitometry was determined with ImageJ version 1.42q software (National Institutes of Health) with normalization to the corresponding controls (β-actin or input).

Statistical Analysis—All assays were repeated at least three times. The results of quantitative studies are reported as mean ± S.D. Differences were analyzed by Student's t test. $p <$
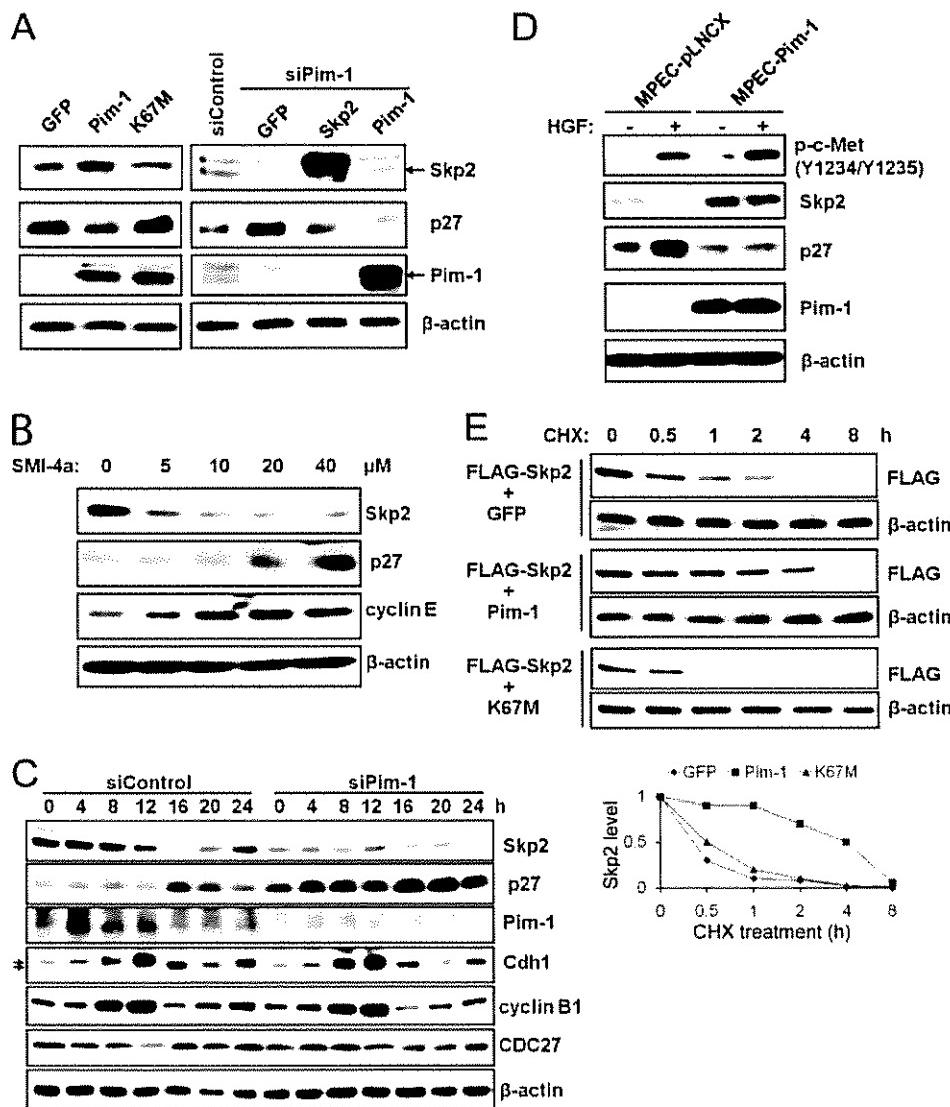
FIGURE 1. **Regulation of Skp2 protein levels by Pim-1.** *A,* HeLa cells were transiently transfected with cDNAs encoding green fluorescent protein (*GFP*), Pim-1, kinase-dead Pim-1 (*K67M*), or a siRNA to Pim-1 together with GFP, or Skp2, or Pim-1, or a scrambled sequence. Forty-eight h after transfection, extracts of these cells were probed on Western blots with the listed antibodies. *B,* HeLa cells were treated with various concentrations of Pim kinase inhibitor SMI-4a for 16 h, extracts were prepared, and immunoblotting was carried out with the identified antibodies. *C,* HeLa cells were transfected with the indicated siRNA plasmids followed by a double-thymidine block treatment (see "Experimental Procedures"). Lysates were prepared at the indicated time points after release from the thymidine block and subjected to immunoblot analysis. *Arrows* indicate phosphorylated and unphosphorylated forms of Cdh1. *D,* mouse prostate epithelial cells (*MPECs*) stably transfected with a control vector (pLNCX) or a human Pim-1-expressing plasmid were treated with HGF (50 ng/ml) for 24 h followed by immunoblot analysis. *E,* 24 h after transfection with expression plasmids (time 0), HEK293T cells were incubated for the indicated times with cycloheximide (*CHX*, 100 µg/ml) followed by immunoblot analysis with FLAG or β-actin antibodies. Densitometric analysis was performed using ImageJ software to quantify the expression of Skp2. Skp2 band intensity was normalized to β-actin, then normalized to the *t* = 0 controls.

targets p27 for proteasomal degradation and in particular the Skp2 protein which is known to directly bind p27. Western blots demonstrate that transfection of the Pim-1 kinase increases the levels of Skp2 protein (Fig. 1*A*), while conversely siRNA or shRNA (supplemental Fig. S1*A*) knockdown of endogenous Pim-1 expression reduces Skp2 levels. The interplay between these two proteins is further demonstrated by the observation that transfection of murine Pim-1 into HeLa cells in which endogenous enzyme has been knocked down again elevates the level of Skp2 (Fig. 1*A*). Using two small molecule Pim kinase inhibitors, SMI-4a, which has demonstrated excellent selectivity (16, 17, 41), and a structurally unrelated Pim kinase inhibitor, K00135 (27), treatment of both HeLa cells (Fig. 1*B*) and PC3 prostate cancer cells (supplemental Fig. S1, *B* and *C*) causes a dose-dependent reduction of Skp2 protein expression and a concomitant rise in p27. We and others have shown that Pim-1 facilitates cell cycle progression as overexpression of Pim-1 promotes G_1-S transition (15) whereas Pim kinase inhibitor caused cell cycle arrest at G_1 (16). Because the Akt protein kinase family is thought to control the level of Skp2 (28, 29), we evaluated whether the PI3K inhibitor, wortmannin or a pan-Akt inhibitor, GSK690693, had similar affects on Skp2 levels. However, no significant changes in the levels of Skp2 were seen after treatment with these reagents until the highest concentrations tested (supplemental Fig. S1, *D* and *E*). Interestingly, LY294002, which is both a PI3K and Pim-1 inhibitor (9),

0.05 was regarded as significant, and such differences are indicated in the figures.

RESULTS

Pim-1 Stabilizes Skp2 Protein—Overexpression in HeLa cells of wild type Pim-1 but not a kinase-dead mutant, K67M, leads to a decrease in the level of the p27 protein (Fig. 1*A*) without any change in the mRNA level of this protein (supplemental Fig. S2*D*). To evaluate the mechanism by which Pim-1 functions, we focused attention on the E3 ligase SCF complex that

reduced Skp2 expression (supplemental Fig. S1*E*).

To test whether the effects of Pim-1 knockdown were cell cycle-specific, we transfected HeLa cells with Pim-1 siRNA, blocked them in the G_1/S boundary, and then released them into the cell cycle and measured the Skp2 and p27 levels. We found that the siRNA knockdown of Pim-1 regulated these two proteins throughout the cell cycle (Fig. 1*C*).

To test the activity of Pim-1 in a different cellular system we examined the role of Pim-1 overexpression in mouse prostate epithelial cells. These cells respond to hepatocyte growth factor

A

FLAG-Skp2: +   +   +
HA-Pim-1:   +   +
HA-K67M: +   +

IP: HA

IB: FLAG
IB: Pim-1

Cell Lysates

IB: FLAG
IB: Pim-1

B

15%FBS: 0   8   16   24   28 h

IP: Pim-1

IB: Pim-1
IB: Skp2

Cell Lysates

IB: Pim-1
IB: Skp2

C

input   6×His-Pim-1   6×His-Ubc3

6×His-Ubc3
6×His-Pim-1
GST
GST-Skp2
GST-Skp2
GST-Skp2
GST

IB: His tag

← GST-Skp2

← GST

D

HA-Skp2:   +   +   +   +
6×His-Ub: +   +   +
FLAG-Pim-1:   +
FLAG-K67M: +

kDa
250 –
130 –
95 –
72 –
55 –

Ni-NTA
IB: HA

Skp2 (HA)
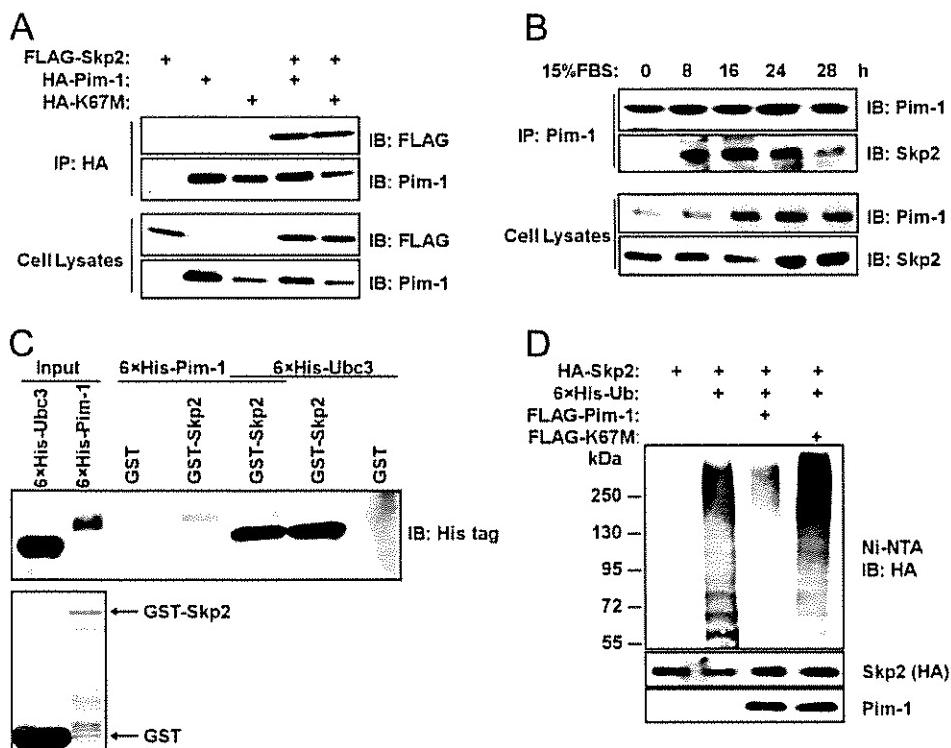
Pim-1

FIGURE 2. **Pim-1 binds to Skp2.** *A*, HEK293T cells were transfected with the indicated plasmids, protein immunoprecipitated (*IP*) with HA antibody and immunoblotted with FLAG or Pim-1 antibody. Lysates of cells used for this assay are probed with identical antibodies. *B*, HEK293T cells were serum-starved (0.2%) for 48 h prior to the addition of 15% FBS at 0 time. Cells were then harvested at the indicated time points, and coimmunprecipitation (*co-IP*) was performed. *C*, GST-Skp2 proteins were incubated overnight with His-tagged Pim-1 or Ubc3 proteins purified from *E. coli* at 4 °C, washed with PBS, and subjected to immunoblot analysis (*upper panel*). GST and GST-Skp2 were stained with Coomassie Brilliant Blue (*lower panel*). *D*, HEK293T cells were transfected with the indicated plasmids, treated with 10 μM MG132 for 6 h, and ubiquitination as measured by binding to nickel-nitrilotriacetic acid (*Ni-NTA*) beads (see "Experimental Procedures") followed by an immunoblot with anti-HA antibody. Immunoblot (*IB*) analysis was performed on total cell lysates from these HEK293T cells (*two lower panels*).

cell cycle analysis on these cells. However, knockdown of endogenous Pim-1 in HeLa cells appears to reduce Skp2 expression throughout a full cell cycle (Fig. 1*C*). These observations suggest that Pim-1 may regulate other molecule(s) controlling Skp2 levels *in vivo*. This binding is also seen *in vitro* in glutathione *S*-transferase (GST) pull-down experiments. Recombinant His-tagged Pim-1 protein binds to Skp2; the binding of Pim-1 did not interfere with the interaction between Skp2 and Ubc3, an E2 ubiquitin enzyme that is known to interact with the Skp2 protein (Fig. 2*C*). Furthermore, Pim-1 did not interfere with the formation of the SCF^{Skp2} complex, from Skp2, Ubc3, and Rbx1 proteins (supplemental Fig. S2*A*).

Like p27, Skp2 levels are regulated by ubiquitination and proteasome degradation (25, 26), suggesting that Pim-1 could decrease the levels of Skp2 ubiquitination. Using protein extracts from HEK293T cells transfected with Pim-1 and Skp2, we found that the presence of active but not kinase-dead Pim-1 is sufficient to repress the ubiquitination of the Skp2 protein markedly (Fig. 2*D*). Using this same approach,

(HGF), a powerful mitogen and morphogen for epithelial and endothelial cells, through binding to its receptor the Met tyrosine kinase (42, 43). The growth inhibitory activity of HGF on cancer cells is associated with up-regulation of p27 expression (44), mediated by down-regulation of Skp2 expression (45). We found that the HGF-induced p27 up-regulation is inhibited by Pim-1 in mouse prostate epithelial cells (Fig. 1*D*). Similar results were also obtained in Pim-overexpressing HeLa cells when they were treated with HGF (supplemental Fig. S1*F*).

Finally, in HEK293T cells the coexpression of Pim-1, but not kinase-dead Pim-1, K67M, or GFP, was able to induce a longer Skp2 half-life (Fig. 1*E*). Taken together, these experiments suggest that Pim-1 controls the levels of Skp2 and consequently regulates the amounts of p27 protein in cells.

Pim-1 Binds Directly to Skp2 and Reduces Skp2 Ubiquitination—We cotransfected HEK293 cells with FLAG-Skp2 and either HA-Pim-1 or kinase-dead Pim-1 (HA-K67M) expression constructs. When cell lysates were subjected to immunoprecipitation with HA antibody, we found that Pim-1 and Skp2 are able to interact physically in cells irrespective of the Pim-1 kinase activity (Fig. 2*A*). In HEK293T cells that are transfected then serum-starved and finally released into 15% serum, this interaction between Pim-1 and Skp2 occurs maximally between hours 8 and 24 (Fig. 2*B*). We did not perform a

consistent with the effect on Skp2, Pim-1 transfection was found to also increase p27 ubiquitination (supplemental Fig. S2*C*). This suggests that an increase in Skp2 levels is needed to mediate increased ubiquitination of p27 by Pim-1. Indeed, in an *in vitro* assay, the presence of Pim-1 did not directly influence p27 ubiquitination (supplemental Fig. S2*B*).

Pim-1 Phosphorylates Skp2 on Multiple Sites—To determine whether Skp2 was a substrate for Pim-1, purified His-tagged protein kinase was incubated with immunoprecipitated FLAG-Skp2 in the presence of [γ-^{32}P]ATP. In this assay, Skp2 was clearly phosphorylated, and this phosphorylation was decreased by the addition of a small molecule Pim-1 inhibitor, SMI-4a (Fig. 3*A*). Pim-1 is known to phosphorylate the sequence R-*X*-R-L-S/T (46). Scanning the Skp2 sequence, we identified a potential Pim-1 consensus site at the C terminus of Skp2, Thr^{417}, which is conserved from frog to humans (Fig. 3*B*). Mutation of this residue from threonine to alanine (T417A) led to reduced Skp2 phosphorylation by Pim-1 *in vitro*, but did not completely abolish this modification (Fig. 3*C*). Previous studies (8–10) have demonstrated that Ser^{64} and Ser^{72} in Skp2 are CDK2 phosphorylation sites (27), and Ser^{72} can also be phosphorylated by Akt1 (28, 29). Using GST-Pim-1 as a kinase, mutation of either Ser^{64} or Ser^{72} to Ala markedly decreased Skp2 phosphorylation by Pim-1 with both of these changes hav-
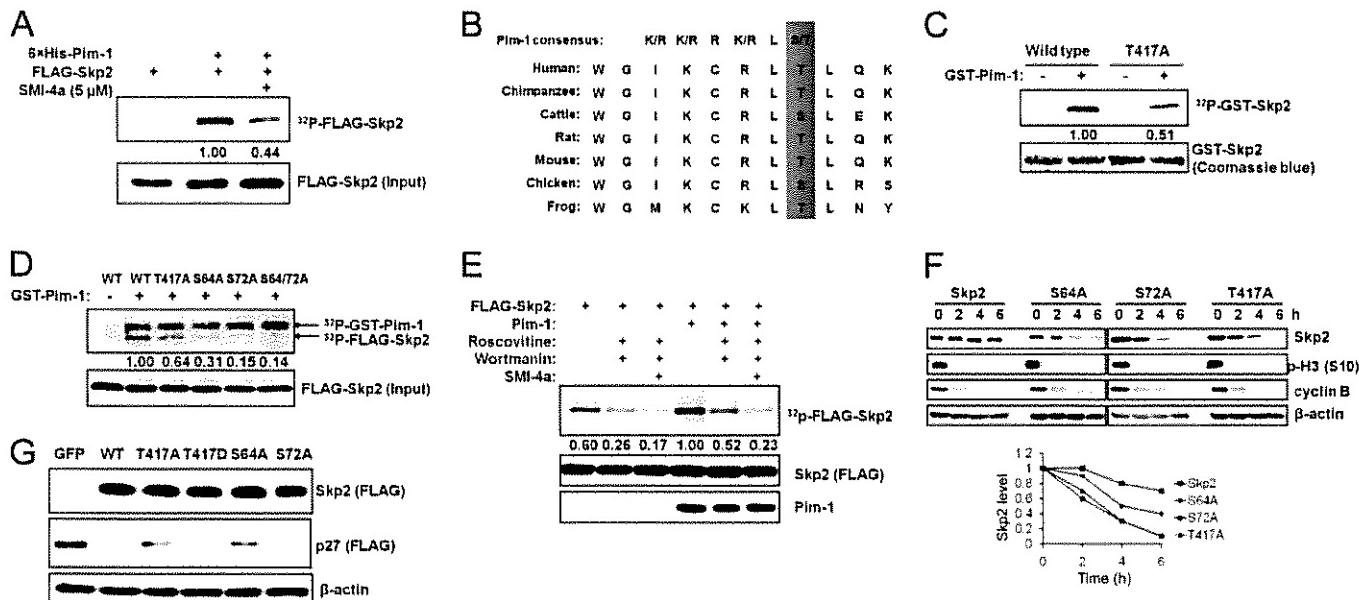
FIGURE 3. **Pim-1 phosphorylates Skp2.** *A*, FLAG-Skp2 was immunoprecipitated from HEK293T cells, incubated with recombinant His-tagged Pim-1 for 30 min with or without SMI-4a for an *in vitro* kinase assay ("Experimental Procedures") followed by SDS-PAGE autoradiography (*upper panel*) and immunoblot analyses (*lower panel*). The phosphorylation of FLAG-Skp2 was quantified by densitometry from three independent experiments after normalization to input. *B*, C-terminal sequence of Skp2 contains a Pim-1 consensus site. *C*, GST-tagged Skp2 proteins or a T417A mutant was incubated with recombinant GST-Pim-1 and [γ-^{32}P]ATP for 30 min, and subjected to SDS-PAGE followed by autoradiography. The phosphorylation of GST-Skp2 was quantified by densitometry from three independent experiments with normalization to Coomassie Blue staining. *D*, wild type (*WT*) FLAG-Skp2 and its mutants T417A, S64A, S72A, and S64A/S72A were immunoprecipitated from HEK293T cells, incubated with recombinant GST-tagged Pim-1 and [γ-^{32}P]ATP for 30 min, followed by SDS-PAGE autoradiography (*upper panel*) and immunoblot analysis (*lower panel*). The phosphorylation of FLAG-Skp2 was quantified by densitometry from three independent experiments following normalization to the level of protein input. *E*, HeLa cells were pretreated with roscovitine (20 μM), wortmannin (1 μM), or SMI-4a (10 μM) for 1 h, transfected with human Pim-1 and Skp2, and labeled with ^{32}P, followed by FLAG immunoprecipitation, autoradiography (*upper panel*), and FLAG/Pim-1 immunoblots (*two lower panels*). The phosphorylation of FLAG-Skp2 was quantified by densitometry from three independent experiments along with normalization to Skp2 expression. *F*, HeLa cells were transfected with the indicated Skp2 constructs and synchronized in M phase by mitotic shake-off of cells obtained after release from a thymidine-nocodazole block. The cells were then replated and allowed to progress through the cell cycle in the presence of cycloheximide (100 μg/ml). Immunoblot analysis was performed at specific time points using antibodies to cyclin B and phosphohistone H3 Ser^{10} (*p-H3 (S10)*) as controls. Densitometric analysis was performed using ImageJ software to quantify the expression of Skp2. Skp2 band intensity was normalized to β-actin and then normalized to the *t* = 0 controls. *G*, HEK293T cells were transfected with a FLAG-tagged p27 Skp2 construct or a GFP control. Expression of exogenous p27 and Skp2 is measured by immunoblotting.

ing a somewhat greater effect than the T417A mutation (Fig. 3*D*), suggesting that each of these sites might also be a Pim-1 target. It appears that phosphorylation of Ser^{64} and/or Ser^{72} may be required for Thr^{417} phosphorylation to take place because mutation of either Ser^{64} or Ser^{72} almost completely abolished Skp2 phosphorylation in this experiment. However, a complete understanding of the relationship between these sites requires further studies.

To test whether Pim-1 has a role in regulating Skp2 phosphorylation *in vivo*, HeLa cells were transfected with Pim-1 and Skp2, metabolically labeled with orthophosphate, and then treated with kinase inhibitors such as roscovitine (pan-CDK inhibitor), wortmannin (PI3K inhibitor), and SMI-4a (Pim-1 inhibitor). Treatment with roscovitine and wortmannin reduced Skp2 phosphorylation *in vivo*. Overexpression of Pim-1 markedly increased Skp2 phosphorylation, and this phosphorylation was inhibited by all three agents (Fig. 3*E*), suggesting that multiple kinases can play a role in regulating phosphorylation of this protein.

Skp2 is a degraded by the APC/C^{Cdh1} (25, 26) which is known to have its highest activity from late mitosis to the G$_1$ phase of the cell cycle (47). To test the impact of phosphorylation of Skp2 on protein stability, we used HeLa cells that were released from a thymidine-nocodazole block in the G$_1$ phase of the cell

cycle into media containing cycloheximide. Exit from mitosis was monitored by the loss of histone H3 phospho-Ser^{10} immunoreactivity and the degradation of cyclin B1 on Western blots (Fig. 3*F*). Using this technique, we found that all three individual Skp2 phosphorylation mutants were more efficiently degraded than the wild type Skp2 protein (Fig. 3*F*), suggesting that phosphorylation by protein kinases, including Pim-1, controls the rate of degradation of Skp2.

We next examined the biological activity of wild type and Skp2 phosphorylation mutants by transfecting them along with p27 into HeLa cells and then examining p27 levels by Western blotting. We found that both the Skp2 T417A and S64A mutants decreased the ability of Skp2 to stimulate the degradation of p27, but T417A retained some degrading activity (Fig. 3*G*). In contrast, an aspartate mutation, T417D, that mimics phosphorylation at this site was more efficient than the T417A at degrading p27. Surprisingly, S72A mutation did not cause any detectable effect on p27 degradation (Fig. 3*G*).

Pim-1 Impairs Cdh1 and CDC27 Interaction and Phosphorylates Cdh1—Because Pim-1 regulates Skp2 ubiquitination, we examined whether this enzyme might interact with components of the APC/C complex that are responsible for Skp2 degradation. In coimmunoprecipitation experiments done in transfected HEK293T cells, Pim-1 was found to complex with
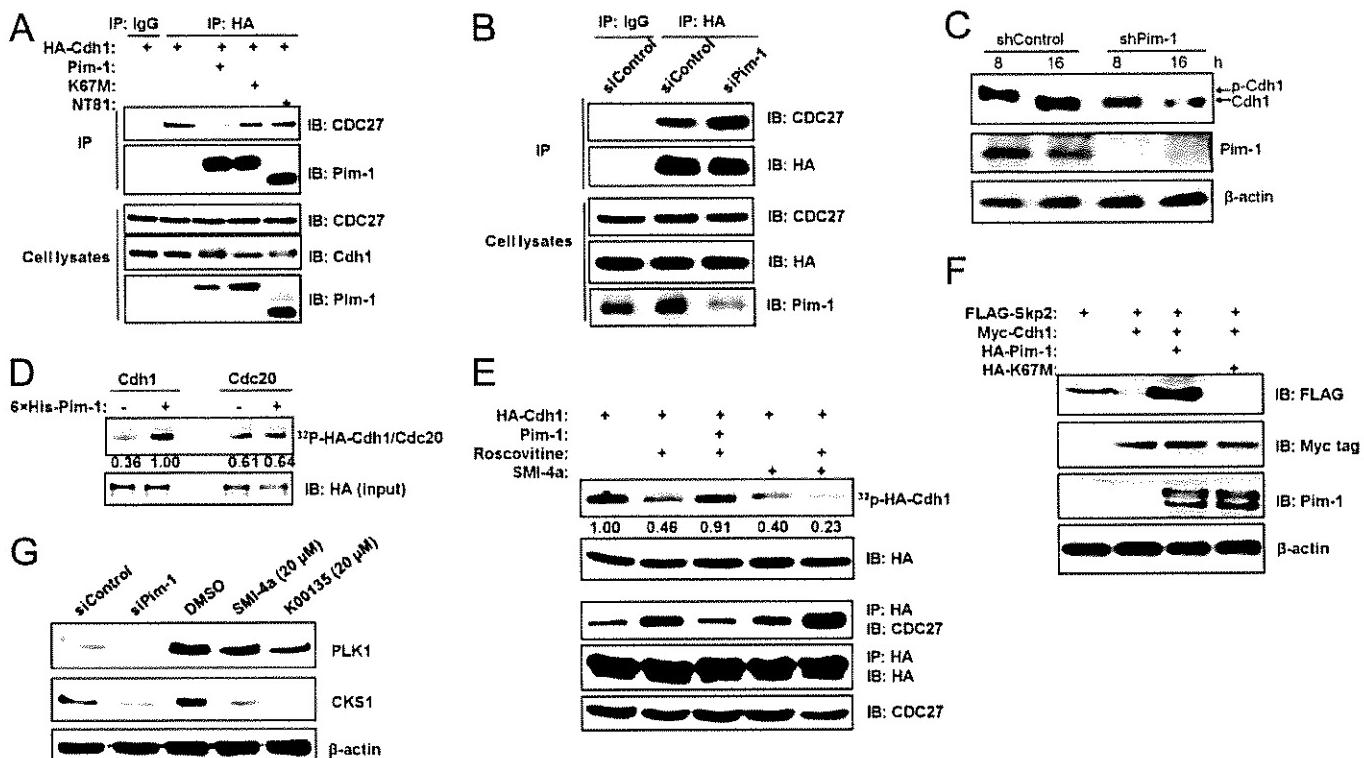
FIGURE 4. **Pim-1 kinase phosphorylates Cdh1 and impairs its binding to CDC27.** *A*, HEK293T cells were transfected with HA-Cdh-1, Pim-1, or kinase-dead Pim-1 K67M or NT81, immunoprecipitated (*IP*) with HA antibody followed by Western blotting with antibodies to CDC27 and Pim-1. Lysates from these cells were immunoblotted (*IB*) with antibody as shown. *B*, HeLa cells were cotransfected with HA-Cdh1 and Pim-1 or scrambled siRNA plasmids before harvesting for coimmunoprecipitation analysis. Levels of transfected proteins in lysates were monitored by immunoblotting. *C*, HeLa cells were transfected with the indicated shRNA plasmids followed by a double-thymidine block. After release from the block cell, lysates were prepared at 8 and 16 h and subjected to immunoblot analysis. The *arrows* indicate phosphorylated and unphosphorylated Cdh-1. *D*, *in vitro* translated HA-tagged Cdh1 or Cdc20 was incubated with recombinant Pim-1 and [γ-^{32}P]ATP in an *in vitro* kinase assay. Autoradiography (*upper panel*) and immunoblot (*lower panel*) analyses were performed. The phosphorylation of Cdh1 or Cdc20 was quantified by densitometry from three independent experiments after normalization to the loaded protein. *E*, HeLa cells were transfected with HA-Cdh1 and human Pim-1, and the kinase inhibitors roscovitine (20 μM) and SMI-4a (10 μM) were added 1 h before labeling with ^{32}P$_i$. HA-Cdh-1 was immunoprecipitated, and autoradiography (*top panel*) and immunoblot analysis were performed (*second panel*). The phosphorylation of Cdh1 was quantified by densitometry from three independent experiments with normalization to Cdh1 expression (HA). A coimmunoprecipitation experiment was performed to monitor Cdh1 and CDC27 interaction under the same experimental conditions (*lower three panels*). *F*, HEK293T cells were transfected with FLAG-Skp2, myc-Cdh-1, and HA-tagged WT and kinase-dead (K67M) Pim kinase, and the extracts were immunoblotted with the specified antibodies. *G*, HeLa cells were transiently transfected with a siRNA to Pim-1 or a scrambled sequence. Forty-eight h after transfection, extracts of these cells were probed on Western blots with the listed antibodies (*left two lanes*). HeLa cells were treated with 20 μM Pim kinase inhibitor SMI-4a or K00135 for 16 h, extracts were prepared, and immunoblotting was carried out with the identified antibodies (*right three lanes*).

either Cdh1 or CDC20, two well known activators of APC/C (supplemental Fig. S3*A*). However, Pim-1 did not physically impair the interaction between Cdh1 or CDC20 and Skp2 (supplemental Fig. S3*B*). Using the same methodology, in contrast, we found that Pim-1 could impair the interaction between Cdh1 and CDC27, another APC/C component, in a phosphorylation-dependent manner (Fig. 4*A*). The two kinase-dead Pim-1 mutants, K67M and NT81 (36), are also able to form a complex with Cdh1, but only wild type Pim-1 was capable of reducing the interaction between Cdh1 and CDC27 (Fig. 4*A*). Additionally, incubation with the Pim-1 inhibitor, SMI-4a (supplemental Fig. S4*C*) or treatment with siRNA to knock down endogenous Pim-1 expression (Fig. 4*B*) increased the Cdh1/CDC27 interaction.

Our results are consistent with previous findings that demonstrate that phosphorylation of Cdh1 dissociates this protein from the APC/C complex (48, 49). Cdh1 is hyperphosphorylated *in vivo* during S, G$_2$, and M phases, and this phosphorylation causes an electrophoretic mobility shift on SDS-polyacrylamide gels (Fig. 5*A*) (48, 50, 51). To explore the role of

Pim-1 in phosphorylation of Cdh1 further, we first reprobed the same membrane used in Fig. 1*C* with antibodies against Cdh1 and CDC27. In cells treated with Pim-1 siRNA, Cdh1 displayed higher mobility at 0, 4, 8, and 12 h compared with those in control cells (Fig. 1*C*), suggesting that phosphorylation was reduced. To confirm this finding, we knocked down endogenous Pim-1 expression in HeLa cells using shRNA and then subjected these cells to a double-thymidine block. The cells were then released from the block (for cell cycle analysis, see Fig. 5*A*), and immunoblotting was performed to examine Cdh1 phosphorylation. Phosphorylation of endogenous Cdh1 at 8 h after release from a double-thymidine block was dramatically reduced in Pim-1 knockdown cells as judged by the protein mobility shift (Fig. 4*C*).

Additionally, we found that recombinant Pim-1 was capable of phosphorylating *in vitro* translated Cdh1 but not CDC20 (Fig. 4*D*). Cdh1 is heavily phosphorylated by CDKs *in vivo*. To examine whether Pim-1 can also phosphorylate Cdh1 *in vivo*, we first treated HeLa cells with the CDK inhibitor roscovitine or Pim inhibitor SMI-4a and then labeled them with ^{32}P$_i$.
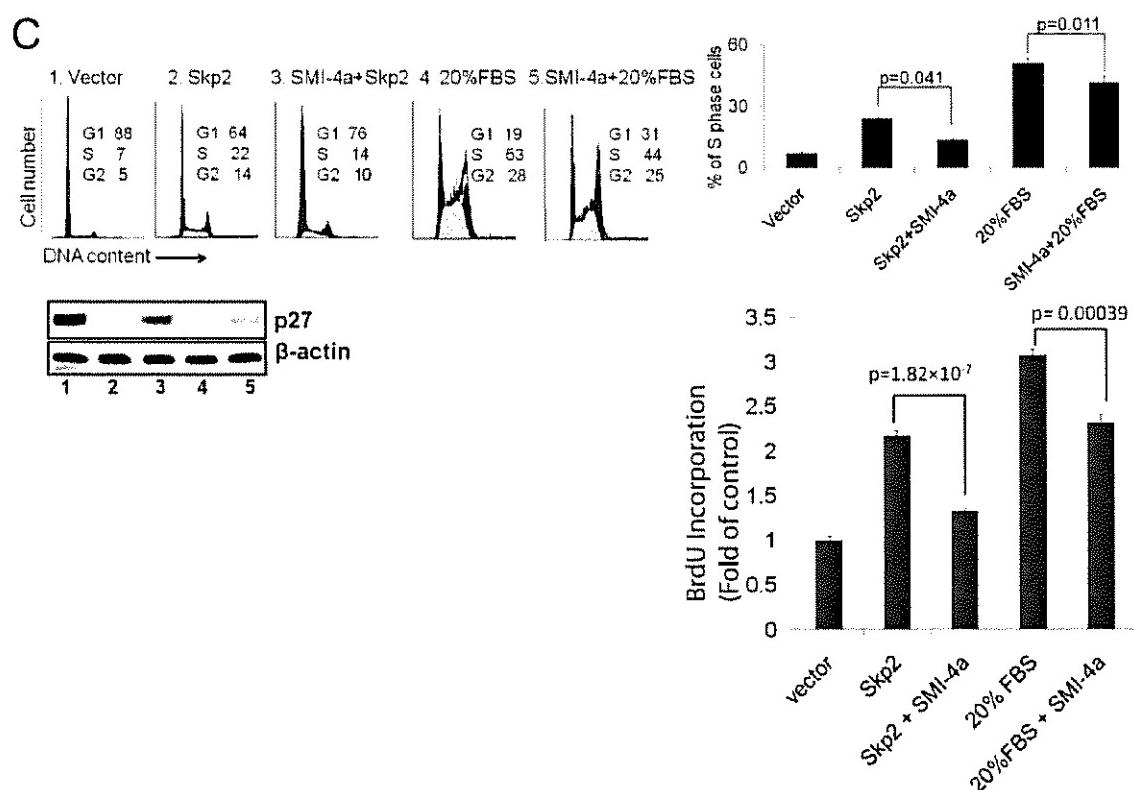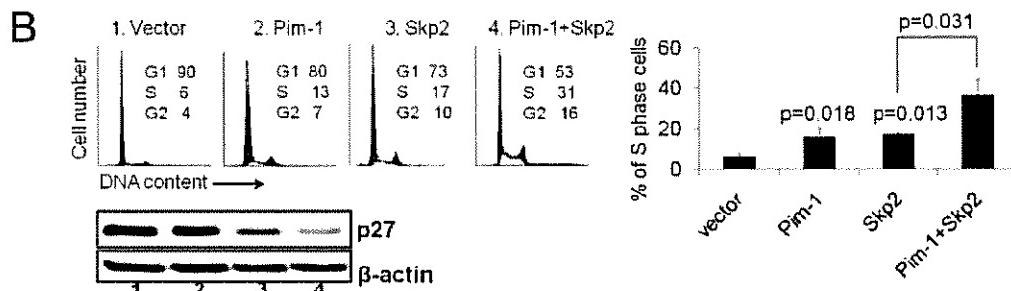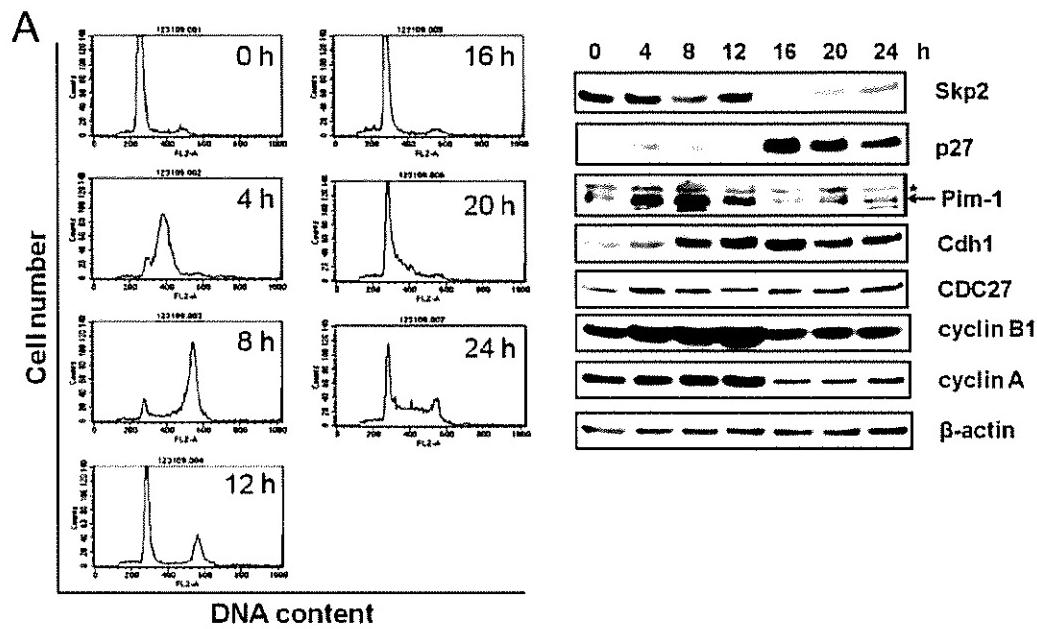
Roscovitine or SMI-4a treatment reduced Cdh1 phosphorylation, and overexpression of Pim-1 in the presence of roscovitine reversed the roscovitine effect, although the combination of roscovitine and SMI-4a treatment further decreased Cdh1 phosphorylation (Fig. 4E). We performed coimmunoprecipitation experiments to monitor the Cdh1/CDC27 interactions under these conditions. Consistently, roscovitine treatment increased the Cdh1/CDC27 interaction whereas overexpression of Pim-1 in the presence of roscovitine suppressed this effect. Combined treatment with roscovitine and SMI-4a further increased the Cdh1/CDC27 interaction compared with roscovitine or SMI-4a alone (Fig. 4E).

Because the APC/C activity to degrade Skp2 can be activated by Cdh1 overexpression (25, 26), we tested the ability of Pim-1 to reverse this effect. We found that coexpression of wild-type Pim-1, but not its mutant, K67M, is capable of blocking Cdh1-mediated degradation of Skp2 (Fig. 4F). However, data in Fig. 4F cannot distinguish whether Pim-1 blocks degradation of Skp2 by acting on Cdh1 or Skp2 or both.

Because Pim-1 expression impairs APC/C$^{\text{Cdh1}}$ activity, we examined whether other known APC/C$^{\text{Cdh1}}$ substrates are regulated by Pim-1 expression. Knockdown of endogenous Pim-1 expression or suppression of Pim-1 kinase activity with Pim kinase inhibitors SMI-4a or K00135 in HeLa cells led to reduced protein expression of both polo-like kinase-1 and CDK subunit 1 (Cks1), two proteins known to be regulated by APC/C$^{\text{Cdh1}}$ (Fig. 4G). This finding further demonstrates that Pim-1 is a negative regulator of APC/C$^{\text{Cdh1}}$ activity.

Pim-1 Is Required for Skp2 to Signal Cell Cycle S Phase Entry—Based on the activities of Pim-1, we have attempted to correlate the levels of this enzyme with other cell cycle regulatory components. HeLa cells were released from a double-thymidine block, cell cycle progression was monitored by FACS analysis (Fig. 5A), and the expression patterns of Skp2, p27, Cdh1, CDC27, cyclin B1, and cyclin A were measured by Western blotting (Fig. 5B). We found that Pim-1 levels were very high at S (4 h) and G$_2$/M phases (8 h) of the cell cycle. Lower Pim-1 expression was seen at G$_1$ and the G$_1$/S boundary (0, 16, 20, and 24 h; Figs. 5A and 1C). Because Pim-1 is a constitutively active kinase (7–11), this expression pattern of Pim-1 should represent its activity profile during cell cycle progression. Interestingly, but not surprisingly, Pim-1 activity coincides with Skp2 expression (Fig. 5A) and inversely correlates with Cdh1 activity (47, 52) during the cell cycle (Fig. 5A). Because Skp2 is known to have the ability to induce S phase in quiescent fibroblasts (40, 53), we determined whether the Skp2/Pim-1 interaction is important for S phase progression. Investigation of this question was carried out using Rat1 cells because they were able to undergo complete cell cycle blockade at G$_0$/G$_1$ upon serum starvation. As judged by FACS analysis, we found that in the absence of serum addition, overexpressed Skp2 or Pim-1 each stimulates S phase entry (Fig. 5B), and coexpression of Skp2 and

Pim-1 further enhances the S phase entry of these cells (Fig. 5B). Conversely, as revealed by both FACS analysis and BrdU incorporation, treatment with a small molecule Pim inhibitor, SMI-4a, reduced Skp2-induced S phase progression (Fig. 5C) and impaired serum-induced S phase entry (Fig. 5C). Another structurally unrelated small molecule Pim inhibitor, K00135 (54), displayed a similar effect (supplemental Fig. S5A). These observations suggest that Pim kinases are required along with Skp2 to allow cells to exit from quiescence.

DISCUSSION

The data presented suggest the novel observation that the Pim-1 protein kinase through a dual mechanism can regulate the levels and hence the activity of Skp2. Pim-1 is capable of binding and phosphorylating Skp2 and stabilizing protein levels, but does not affect the interaction of Skp2 with the E2 ligase Ubc3. Conversely, both siRNA and small molecule Pim-1 inhibitors decrease Skp2 levels and phosphorylation. Skp2 is phosphorylated by CDK2 at Ser^{64} and Ser^{72} (27) and by Akt1 at Ser^{72} to stabilize this protein (28, 29). Pim-1 appears capable of phosphorylating Skp2 at these two sites (Fig. 3), as well as a unique site in the C terminus, Thr^{417}, that is highly conserved throughout the animal kingdom, including humans and mice. Phosphorylation of this site is required for maximal Skp2 activity and stabilization of Skp2 protein levels *in vivo* (Fig. 3). In the prostate cancer cell line PC3 that contains an activated Akt, a small molecule Pim inhibitor SMI-4a but not wortmannin or the Akt inhibitor GSK690693 decreased the levels of Skp2. LY294002, which inhibits both Akt and Pim, displayed an effect similar to that of SMI-4a, suggesting that in this cell line the Pims are essential for the regulation of Skp2 levels. Unlike Akt (28, 29), Pim-1 kinase did not appear to regulate Skp2 subcellular localization (supplemental Fig. S4). The Pim kinases share multiple similarities with AKT (1, 55, 56). It is possible that the relative abundance of each of these Skp2-phosphorylating kinases may decide which is essential to the control of Skp2 levels. It is quite surprising that our Skp2 S72A mutant did not lose p27 degradation activity compared with the wild type Skp2 (Fig. 3G) because two previous studies demonstrated that this very same Skp2 mutant completely lost ubiquitin ligase activity (28, 29). However, another two recent reports confirmed our finding (30, 31). The half-life of this mutant was indeed shorter than that of wild-type Skp2 (Fig. 3F), consistent with previous reports (27–29).

The degradation of Skp2 is regulated by APC/C$^{\text{Cdh1}}$ complex (25, 26) which preferentially associates with non-phospho-Ser^{64} form of Skp2 (27). Pim-1 kinase activity does not affect the binding of Cdh1 to total Skp2 (supplemental Fig. S3B), but does impair the interaction between Cdh1 and CDC27 (Fig. 4A). Interaction with CDC27/APC3 protein allows Cdh1 to activate the APC/C (57). Although Cdh1 is inhibited by both the Emi-1 protein and multiple phosphorylations initiated in part by

FIGURE 5. **Pim-1 is required for Skp2 to signal cell cycle S phase entry.** *A*, HeLa cells were treated with a double-thymidine block and released into fresh medium. Cells were then harvested at the indicated time points and subjected to FACS (*left panel*) and immunoblot analysis (*right panel*). The *arrow* denotes the Pim-1 signal. * indicates a nonspecific signal. *B*, Rat1 cells were transduced with a lentivirus carrying the indicated cDNAs. Cells were maintained in low serum conditions (0.2%) for 48 h before harvested for FACS (*upper panel*) and immunoblot (*lower panel*) analyses. Percent of S phase cells was compared with vector control, except where indicated by a *bracket*. *C*, the experiment was performed as in *B* except that SMI-4a (5 μM) was added 3 h before a 20% FBS stimulation (16 h). S phase induction was also determined by BrdU incorporation assay. *Brackets* indicate comparison of with and without SMI-4a treatment.
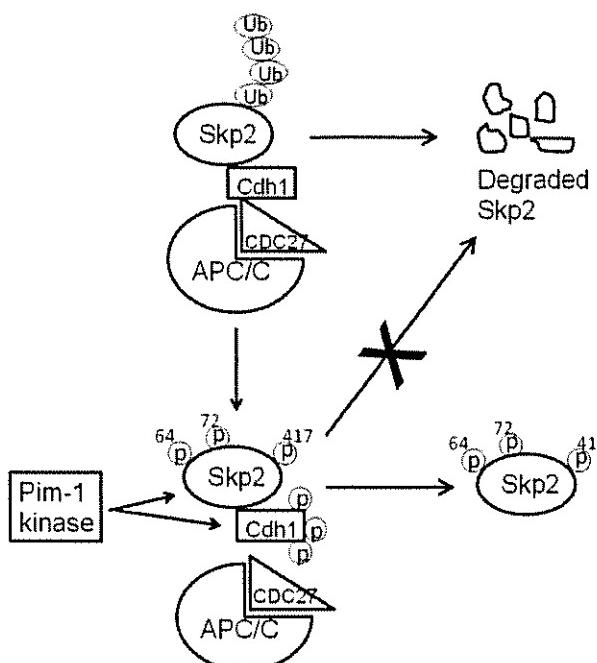
FIGURE 6. Model of Pim-1 regulation on Skp2 degradation. Nonphosphorylated Skp2 binds to E3 ligase APC/C^{Cdh1} and gets ubiquitinated (Ub) followed by proteasome-mediated degradation. Pim-1 kinase phosphorylates Skp2 on multiple sites: Ser^{64}, Ser^{72}, and Thr^{417}. Furthermore, the phosphorylation of Cdh1 by Pim-1 reduces Cdh1 and APC/C interaction. Both Pim-1 actions result in decreased Skp2 ubiquitination and consequently increased Skp2 stability.

cyclin A-CDK2 and cyclin B1-CDK1 (47, 52), it has been proposed that an additional kinase may play a role (58). Here, we demonstrate that Cdh1 is a phosphorylation target of Pim-1 (Fig. 4, *D* and *E*) and the knockdown of Pim-1 with siRNA reduces Cdh1 phosphorylation during S, G$_2$, and M phases (Figs. 1*C* and 4*C*), demonstrating the critical involvement of Pim-1 in tightly controlled Cdh1 phosphorylation during the cell cycle. It remains unknown whether Pim-1 and CDKs share some phosphorylation sites on Cdh1. Further studies are required to determine the precise Pim-1 sites and how these two different types of kinases cooperate to control Cdh1 activity. Interestingly, the levels of Pim-1 protein are correlated with Cdh1 phosphorylation during cell cycle progression as high Pim-1 expression and high Cdh1 phosphorylation were seen during S, G$_2$, and M phases, and the opposite occurred during the G$_1$ phase (Fig. 5*A*). Given the role of Cdh1 in regulating mitosis, this may explain why Pim-1 is not only required for Skp2 to signal S phase entry (Fig. 5), but also plays a critical role in G$_2$/M phase regulation. Consistent with this hypothesis, mouse embryo fibroblasts that are knocked out for all three Pim kinase isoforms display increased number of cells in the G$_2$/M phase of the cell cycle (supplemental Fig. S5). These observations are in concert with previous discoveries suggesting that Pim-1 functions in mitosis (59–61). Therefore, the Pim-1 kinase regulates Skp2 levels through the Pim-1 kinase activity, reduces APC/C^{Cdh1} E3 ligase activity, and thus protects Skp2 from degradation (Fig. 4*F*).

The Pim-1 protein kinase is abnormally elevated in human cancers, regulated by growth factors, and collaborates with other oncogenes to induce cell transformation (1, 2, 5, 6). The

ability of this enzyme to modulate the activity of both the SCF^{Skp2} and APC/C^{Cdh1} (Fig. 6) and thus control p27 levels is likely to be essential to the biological activities of this protein kinase.

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The Pim protein kinases regulate energy metabolism and cell growth

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The serine/threonine Pim kinases are overexpressed in solid cancers and hematologic malignancies and promote cell growth and survival. Here, we find that a novel Pim kinase inhibitor, SMI-4a, or Pim-1 siRNA blocked the rapamycin-sensitive mammalian target of rapamycin (mTORC1) activity by stimulating the phosphorylation and thus activating the mTORC1 negative regulator AMP-dependent protein kinase (AMPK). Mouse embryonic fibroblasts (MEFs) deficient for all three Pim kinases [triple knockout (TKO) MEFs] demonstrated activated AMPK driven by elevated ratios of AMP:ATP relative to wild-type MEFs. Consistent with these findings, TKO MEFs were found to grow slowly in culture and have decreased rates of protein synthesis secondary to a diminished amount of 5′-cap–dependent translation. Pim-3 expression alone in TKO MEFs was sufficient to reverse AMPK activation, increase protein synthesis, and drive MEF growth similar to wild type. Pim-3 expression was found to markedly increase the protein levels of both c-Myc and the peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α), enzymes capable of regulating glycolysis and mitochondrial biogenesis, which were diminished in TKO MEFs. Overexpression of PGC-1α in TKO MEFs elevated ATP levels and inhibited the activation of AMPK. These results demonstrate the Pim kinase-mediated control of energy metabolism and thus regulation of AMPK activity. We identify an important role for Pim-3 in modulating c-Myc and PGC-1α protein levels and cell growth.

LKB1 | mitochondria | mTOR | 4EBP1

The Pim serine/threonine kinases include three isoforms, Pim-1, Pim-2, and Pim-3, that are implicated in the growth and progression of hematological malignancies, prostate cancer, and, in the case of Pim-3, in precancerous and cancerous lesions of the pancreas, liver, colon, and stomach (1–5). Pim-1 and Pim-2 have been shown to cooperate with c-Myc in inducing lymphoma (6), and prostate cancer (7), and in the absence of Pim-1 and Pim-2, Pim-3 is activated in c-Myc-induced lymphomas (8). The mechanisms suggested to explain this Pim–Myc synergism include Pim-mediated stabilization of c-Myc protein (9) and regulation of gene transcription via Pim-1 phosphorylation of histone H3 at active sites of c-Myc transcription (10). Other Pim kinase substrates that suggest these enzymes play a role in cell cycle progression and antiapoptosis include BAD, Bcl-2, Bcl-xL (11, 12), p27^Kip1 (13), and Cdc25A (14).

Recently, Pim kinases have been suggested to promote the activity of the rapamycin-sensitive mammalian target of rapamycin (mTORC1) (15–17). mTORC1 is a serine/threonine kinase that regulates cell growth and metabolism (18). The mTORC1 complex, composed of mTOR, raptor, GβL, and PRAS40, promotes protein synthesis by phosphorylating 4EBP1, thus stimulating its dissociation from the translational regulator eukaryotic initiation factor 4E (eIF4E) (17) allowing for cap-dependent translation. mTORC1 activity is regulated by a cascade of enzymes including LKB1, AMP-dependent protein kinase (AMPK), and TSC1 and 2 (19). AMPK senses the cellular energy status and is activated via LKB1-mediated phosphorylation when there is a decline in ATP

levels and concomitant rise in AMP levels; i.e., high AMP:ATP ratio (20). Activated AMPK down-regulates the energetically demanding process of protein synthesis by inhibiting mTORC1 activity through phosphorylating TSC2 and raptor (20). The mechanisms by which Pim kinase stimulates mTORC1 appear complex and include 4EBP1, eIF4E (16, 21–23), and PRAS40 phosphorylation (15).

Because of the importance of the Pim kinase signal transduction pathway in the progression of various cancers, multiple groups have developed small-molecule inhibitors of this kinase family (24–28). We have identified unique benzylidene-thiazolidine-2-4-diones (23, 29) that inhibit Pim kinase activity in vitro at nanomolar concentrations, and in culture induce apoptosis of human leukemic cells (30) and synergize with rapamycin to downregulate 4EBP1 phosphorylation and inhibit cell growth (29). Taking advantage of these inhibitors, siRNA, and genetically engineered Pim-deficient cells, we have discovered a unique role for Pim-3 in regulating mTORC1 activity through modulation of ATP levels by the induction of c-Myc and the transcriptional coactivator and master regulator of mitochondrial biogenesis peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α).

Results

Pim Kinase Negatively Regulates AMPK. To examine the mechanisms by which Pim kinase can regulate the mTORC1 pathway, the human erythroleukemia cell line K562 was incubated with the thiazolidinedione Pim kinase inhibitor SMI-4a (23), and the phosphorylation of AMPK was studied. AMPK activation results in the phosphorylation of raptor and TSC2 and thus inhibits mTORC1 activity (20, 31). Pim kinase inhibition with SMI-4a induced the activation of AMPK as determined by phosphorylation of AMPKα at Thr172, and the AMPK targets acetyl-CoA carboxylase (ACC) at Ser79 and raptor at Ser792 and inhibition of mTORC1 activity as determined by decreased phosphorylation of the mTORC1 targets S6K and 4EBP1 (Fig. 1 A and B). Additionally, knockdown of Pim-1 levels with a targeted siRNA increased AMPK phosphorylation (Fig. 1C), suggesting that Pim-1 negatively regulates the phosphorylation of this enzyme. Because the LKB1 kinase is known to activate AMPK via phosphorylation at Thr172 (32) and loss of LKB1 activity is frequently associated with the transformed phenotype (32), we examined the ability of SMI-4a and SMI-16a, another Pim kinase inhibitor, (29) to regulate AMPK phosphorylation in a panel of LKB1-containing (H358, H661) and deficient (H23, H460, A549) lung cancer cell
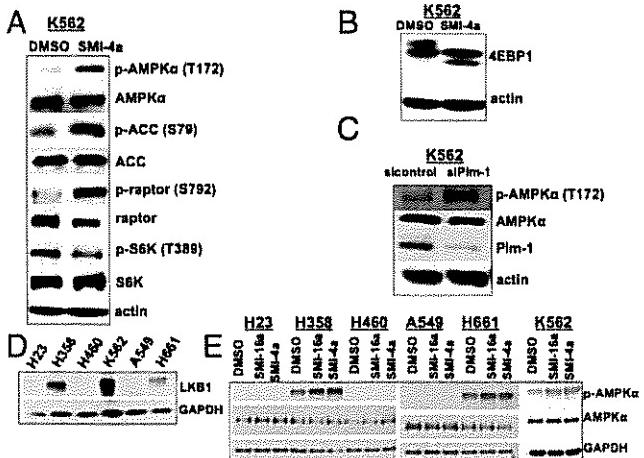
Fig. 1. Pim kinase inhibition activates AMPK. (*A* and *B*) K562 cells were treated with DMSO or SMI-4a (5 μM) for 1 h in the absence of serum, and lysates were probed for the indicated proteins by Western blotting. (*C*) K562 cells were transfected with scrambled siRNA (sicontrol) or Pim-1 siRNA (siPim-1), and 48 h later lysates were probed for the indicated proteins by Western blotting. (*D*) Western blot for LKB1 levels in lung cancer cell lines and the leukemia cell line K562. (*E*) Lung and leukemia cells were treated with DMSO, SMI-4a, or SMI-16a (5 μM) for 1 h in the absence of serum, and lysates were probed for the indicated proteins by Western blotting.

lines along with the LKB1-positive K562 cell line (Fig. 1*D*). These results demonstrate that the Pim kinase inhibitors SMI-4a and SMI-16a required LKB1 to stimulate AMPK activity (Fig. 1*E*).

To further confirm that Pim kinase regulates the activation of AMPK, we generated mouse embryonic fibroblasts (MEFs) deficient for Pim-1, -2, and -3 [triple knockout (TKO)] (33),

and wild-type (WT) littermate control MEFs. Consistent with both the siRNA and small-molecule inhibition of Pim kinase activity, TKO MEFs had significantly higher AMPK phosphorylation compared to WT MEFs (Fig. 2*A*). To determine the contribution of each Pim isoform to AMPK activation, TKO MEFs were transduced with Pim-1, -2, or -3 lentiviruses. Although each Pim isoform reduced p-AMPK levels, Pim-3 showed the greatest effect (Fig. S1*A*) leading us to focus on elucidating the unique role of Pim-3. To confirm this result, we generated MEFs deficient in Pim-1 and Pim-2 (Pim-1$^{-/-}$, -2$^{-/-}$, -3$^{+/+}$) but expressing Pim-3, and demonstrated that these cells showed less activated AMPK than TKO MEFs (Fig. 2*A*). As AMPK activation is regulated by increased AMP, we measured the levels of AMP and ATP and found that AMPK phosphorylation correlates with the cellular AMP:ATP ratio in these knockout MEFs (Fig. 2*B*). Growth curves of these MEFs demonstrated a further correlation between proliferation, AMP:ATP ratio, and AMPK phosphorylation status (Fig. 2*C*) with the TKO MEFs showing the slowest growth rate. Similar results were obtained with immortalized WT or TKO MEFs transduced with empty vector or a lentivirus expressing Pim-3 (Fig. S1*B*).

Because activation of AMPK leads to inhibition of mTORC1 activity (31), we measured the level of protein synthesis in each of the MEFs. Labeling of MEFs with ^{35}S-methionine and measuring newly synthesized protein demonstrated, as predicted, that TKO MEFs when compared to WT have lower rates of protein synthesis (approximately 58% relative to WT). Expression of Pim-3 in the TKO cells increased protein synthesis from 58% (TKO) to 83% relative to WT (Fig. 2*D*). Consistent with this result, we found that in TKO MEFs the cap-dependent but not internal ribosome entry site (IRES)-dependent translational activity is reduced (Fig. 2*E*). Cap-dependent translation depends on the mTORC1-mediated release of 4EBP1 from eIF4E and the for-
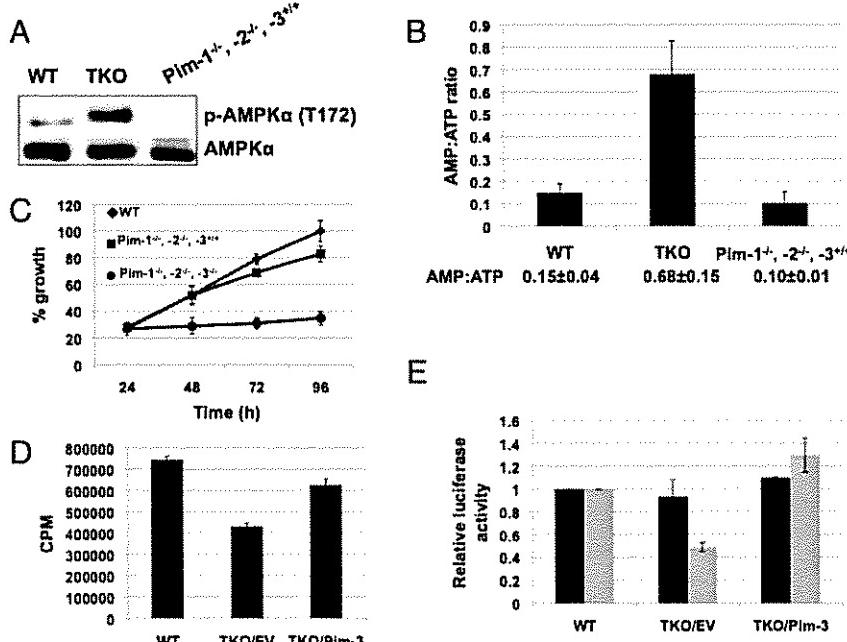

Fig. 2. Knockout of Pim kinase isoforms inhibits protein synthesis and cell growth. (*A*) Lysates were prepared from the different MEF cell lines and probed for the indicated proteins by Western blotting. (*B*) AMP:ATP ratios were determined by HPLC as described in *Materials and Methods*. Values are the average of three independent experiments, and the standard deviation from the mean is shown. (*C*) Growth curve of MEFs as determined by MTT assay. Percentage values are relative to the growth of WT MEFs at the 96 h time point (100%). The data points are the average of three independent measurements, and the standard deviation from the mean is shown. (*D*) ^{35}S-methionine incorporation into WT and TKO MEFs expressing empty vector (TKO/EV) or Pim-3 (TKO/Pim-3). Values (cpm/mg protein) are the average of three independent measurements, and the standard deviation for the mean is shown. (*E*) Cap-dependent (gray bars) and IRES-dependent (black bars) translation in MEFs as measured by Renilla and Firefly luciferase activities, respectively. As described in *Materials and Methods*, MEFs were infected with a virus expressing cap- and IRES-driven luciferase constructs. Values are the ratio of luciferase activity relative to WT and are the average of three independent measurements with the standard deviation from the mean shown.

mation of the eIF4F complex. Using m⁷-GTP beads in a pull-down assay (34), we found that in TKO MEFs eIF4E is highly bound to 4EBP1, whereas eIF4G binding is lost and thus the ability of eIF4E to promote translation is inhibited (Fig. S1C). These results demonstrate that reduced Pim kinase activity correlates with increased AMP:ATP ratio, activation of AMPK, inhibition of mTORC1 activity, and reduced overall protein synthesis.

Pim-3 Regulates c-Myc Levels. In comparison to TKO MEFs, Pim-3–expressing cells demonstrated increased 5′-cap-dependent protein synthesis and growth similar to WT MEFs. Because the levels of the c-Myc protein are controlled by 5′-cap-dependent transcription, and c-Myc is important in the regulation of both cell growth and overall cellular metabolism, we examined the levels of c-Myc in MEFs from each genotype. Western blots demonstrate that the expression of Pim-3 either in primary MEFs (Fig. 3A) or after transduction into TKO MEFs (Fig. 3B) markedly increased c-Myc protein. Similar to p-AMPK (Fig. S1A), neither Pim-1 nor Pim-2 was able to induce the significant levels of c-Myc protein observed with Pim-3 overexpression in TKO MEFs (Fig. 3B). Furthermore, expression of Pim-1 or Pim-2 in Pim-3–only MEFs led to a decrease in c-Myc protein levels (Fig. S1D), suggesting the possibility that the Pim kinases could compete for substrates or interact directly. Because of the marked difference in c-Myc protein levels in MEFs containing Pim-3 only, we tested two additional MEF cell lines generated from different embryos of the same genotype and again observed increased c-Myc protein levels (Fig. S2B). To determine whether the increased c-Myc level in Pim-3–expressing cells is unique to MEFs in culture, we measured the c-Myc levels in spleen lysates of 4-mo-old WT, TKO, and Pim-1⁻/⁻, -2⁻/⁻, -3⁺/⁺ mice and again found the highest level of c-Myc protein in the Pim-3–only genotype (Fig. S2C). Because deletion of one Pim might grossly elevate the level of another, in this case Pim-3, we measured the level of Pim-3 mRNA in the different MEF genotypes but did not find a significant difference (Fig. S2A). These results suggested that Pim-3 might modulate c-Myc translation.

To determine the effect of Pim-3 on c-Myc translation, we treated MEFs with cycloheximide until c-Myc protein was completely degraded, washed out the cycloheximide, and then monitored by Western blotting the rate of increase in c-Myc protein over time. To make this comparison possible, 2.5 times more protein from TKO MEFs was loaded on these SDS gels. TKO MEFs showed a delay in protein synthesis with only 53 and 43% as much c-Myc protein synthesis in the first 15 min when compared to WT and TKO/Pim-3 MEFS, respectively (Fig. 3C). This result is consistent with the reduced protein synthesis in the TKO MEFs (Fig. 2D). Because the overall translational efficiency in cells is reflected by changes in the polysome/monosome ratio, we determined the polysome profile using cytosolic extracts of WT, TKO, and TKO/Pim-3 cells. TKO cells showed a significant reduction in heavy polysomes and a corresponding increase in free ribosome subunits (Fig. 3D Upper). However, expression of Pim-3 in TKO cells resulted in a significant increase in heavy polysomes. c-Myc mRNA showed redistribution from heavy toward lighter polysomes and monosomes and free subunits in TKO cells relative to WT (Fig. 3D Lower). This shift was reversed by Pim-3 expression, suggesting that Pim-3 is capable of controlling the translation of c-Myc mRNA (Fig. 3D Lower). Because Pim-1 and -2 have been shown to increase the stability of c-Myc (9), we examined changes in c-Myc protein stability in TKO and TKO/Pim-3 MEFs after cycloheximide treatment but found no significant difference in c-Myc half-life in the Pim-3–containing cells (Fig. S2D). Finally, we found that the expression of c-Myc in TKO MEFs led to a decrease in AMPK activation (Fig. S2E) consistent with the ability of c-Myc to stimulate cell growth.

Pim-3 and c-Myc Regulate PGC-1α Levels. The differences in the growth rate between the TKO and Pim-3 only MEFs could possibly be explained by the Pim-3–mediated increased c-Myc because the latter is known to control multiple factors that regulate cell growth and metabolism (35, 36). Therefore, we compared the growth rate of TKO MEFs stably expressing empty vector, Pim-3, or c-Myc to WT MEFs. The TKO/c-Myc MEFs were able to
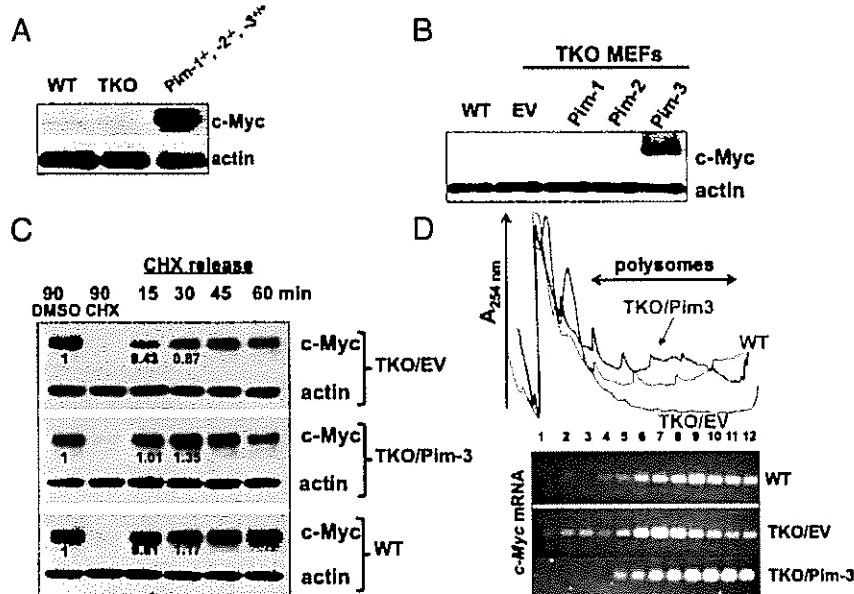

Fig. 3. Pim-3 elevates c-Myc levels. (A) c-Myc protein levels in each MEF genotype as determined by Western blotting. (B) TKO MEFs were infected with empty vector (EV), Pim-1, -2, or -3 lentiviruses, and 48 h later lysates were probed for c-Myc levels and compared to WT MEFs. (C) TKO MEFs expressing EV or Pim-3 were treated for 90 min with cycloheximide (CHX, 10 μM), the media replaced, and lysates probed for c-Myc levels by Western blotting. Densitometry analysis was performed, and the values at the 15 min time point relative to DMSO are shown. To obtain a relatively equal amount of c-Myc protein at the 90 min time point with DMSO, ~2.5-fold more TKO/EV protein lysate was loaded relative to TKO/Pim-3. (D) Ribosome fractions of WT and TKO MEFs expressing EV or Pim-3 were prepared by sucrose gradient (see Materials and Methods), and the level of c-Myc mRNA associated with each fraction was determined by PCR.

grow in the absence of Pim kinases but did not reach the same density in 96 h (Fig. 4A). The shRNA-mediated knockdown of c-Myc in TKO/Pim-3 MEFs did not completely inhibit cell proliferation (Fig. 4B). Together, these results suggest that Pim-3 and c-Myc do not have completely overlapping biologic activities. To understand how Pim-3 decreases the AMP:ATP ratio and inhibits AMPK phosphorylation, we measured the levels of PGC-1α. PGC-1α activates a wide variety of transcription factors that result in increased mitochondrial biogenesis and oxidative phosphorylation (37). Increased expression of PGC-1α can lead to elevations in ATP levels (38), whereas PGC-1α knockout leads to reduced ATP levels in murine hearts (39). PGC-1α expression and PGC-1α–dependent gene expression are induced by chemical activation of AMPK, and AMPK directly phosphorylates PGC-1α, leading to increased transcriptional activity (40–42). We found that the levels of PGC-1α mRNA and protein were greatly reduced in TKO MEFs, highest in Pim-3–only MEFs, and intermediate in WT cells (Fig. 4C and D). To examine the contributions of Pim-3 and c-Myc in regulating PGC-1α levels, we infected TKO MEFs with lentiviruses expressing c-Myc or Pim-3 and found that Pim-3 induced marked increases in PGC-1α mRNA (12-fold) and protein; the effect of c-Myc alone was a 4-fold increase in mRNA, and the increase in protein was quantitated at only 10% that of Pim-3 (Fig. 4D, E).

The above results suggest that the increased AMP:ATP ratio in TKO MEFs may be attributed to low ATP levels due to decreased PGC-1α protein, thus leading to AMPK activation. To examine whether overexpression of PGC-1α in TKO MEFs was sufficient to reduce p-AMPK by increasing the level of cellular ATP, we transduced TKO MEFs with a lentivirus expressing PGC-1α. Western blots and biochemical analysis demonstrate that PGC-1α expression in TKO MEFs decreased the level of p-AMPK (Fig. 5A) and increased the levels of ATP (Fig. 5B), leading to decreased 4EBP1 binding to eIF4E while increasing

eIF4G association with the eIF4E protein (Fig. S3). In contrast, PGC-1α expression in TKO MEFs showed little effect on c-Myc levels (Fig. 5A). Thus, Pim-3, by controlling the levels of both c-Myc and PGC-1α, is able to impact on AMPK phosphorylation, mTORC1 activity, 5'-cap-dependent translation, and ultimately cell growth (Fig. 5C).

Discussion

The combined approach of genetic knockout, RNAi, and small-molecule inhibition implicate the Pim kinases in regulating the AMP:ATP ratio and energy metabolism. These effects lead to the modulation of the mTORC1 pathway by AMPK and the control of cell growth. In leukemic cells, the pan-Pim kinase inhibitor SMI-4a stimulated the phosphorylation and activation of AMPK, whereas in TKO MEFs the ratio of AMP:ATP was markedly increased and AMPK was activated. Because AMPK is a negative regulator of mTORC1, we found in leukemic cells treated with SMI-4a and in TKO MEFs that mTORC1 activity is inhibited and cap-dependent translation is significantly decreased. In MEFs, the expression of Pim-3 alone could reverse these processes, lowering the AMP:ATP ratio, decreasing the activation of AMPK, and increasing cap-dependent translation, all resulting in cellular growth rates comparable to WT MEFs. The differences between the TKO and Pim-3–only MEFs could be explained in part by the Pim-3–mediated increased c-Myc because the latter controls multiple transcription factors that regulate cell growth and metabolism (35, 36). Infection of TKO MEFS with a lentivirus expressing c-Myc increased the growth of these cells but did not duplicate the growth curve of Pim-3–expressing MEFs.

In muscle and fat tissue, the ability of activated AMPK to maintain an energy balance is achieved in part by stimulating PGC-1α (41). The ability of PGC-1α to coactivate multiple transcription factors makes this protein a master regulator of mitochondrial biogenesis (43). Considering this link between
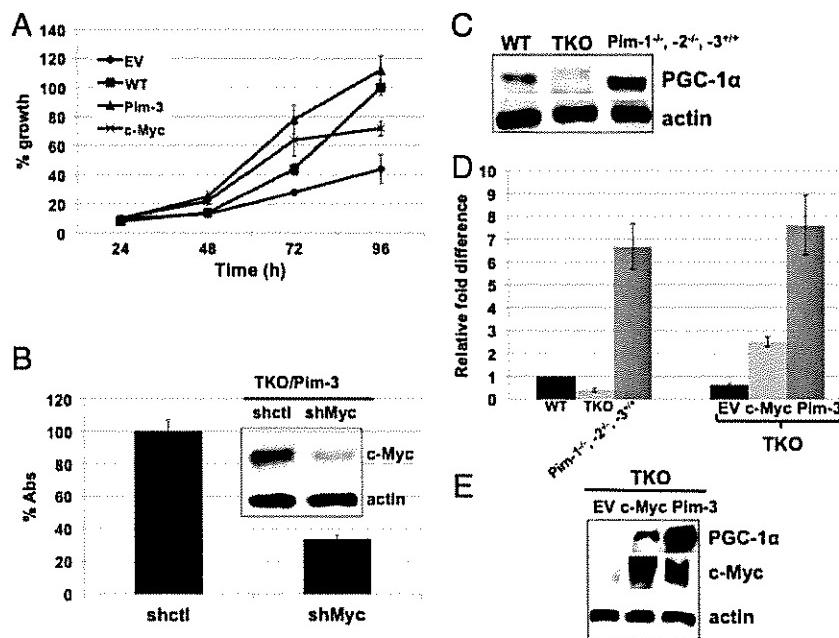
Fig. 4. Pim-3 and c-Myc affect PGC-1α levels. (A) Growth curve of TKO MEFs expressing empty vector (EV), c-Myc, or Pim-3 as determined by an MTT assay. Percentage values are relative to the value of WT MEFs at the 96 h time point (100%). The data points are the average of three independent measurements, and the standard deviation from the mean is shown. (B) TKO/Pim-3 MEFs were infected with nontargeting shRNA (shctl) or c-Myc targeting shRNA (shMyc) lentiviruses. Equal numbers of shctl and shMyc cells were plated 48 h postinfection, and after an additional 72 h viability was determined by an MTT assay and represented as a percent absorbance (%Abs) with shctl set at 100%. The data points are the average of three independent measurements, and the standard deviation from the mean is shown. (Inset) Lysates were prepared at 120 h postinfection and probed for the indicated proteins by Western blotting. (C) PGC-1α protein levels in MEFs as determined by Western blotting. (D) PGC-1α mRNA levels in primary MEFs (WT, TKO, Pim-1⁻/⁻, -2⁻/⁻, -3⁺/⁺) or TKO MEFs infected with EV, c-Myc, or Pim-3 lentiviruses as determined by QT-PCR 48 h after infection. Values are the average of three independent measurements, and the standard deviation from the mean is shown. (E) PGC-1α protein levels as determined by Western blotting in TKO MEFs 48 h postinfection with EV, c-Myc, or Pim-3.
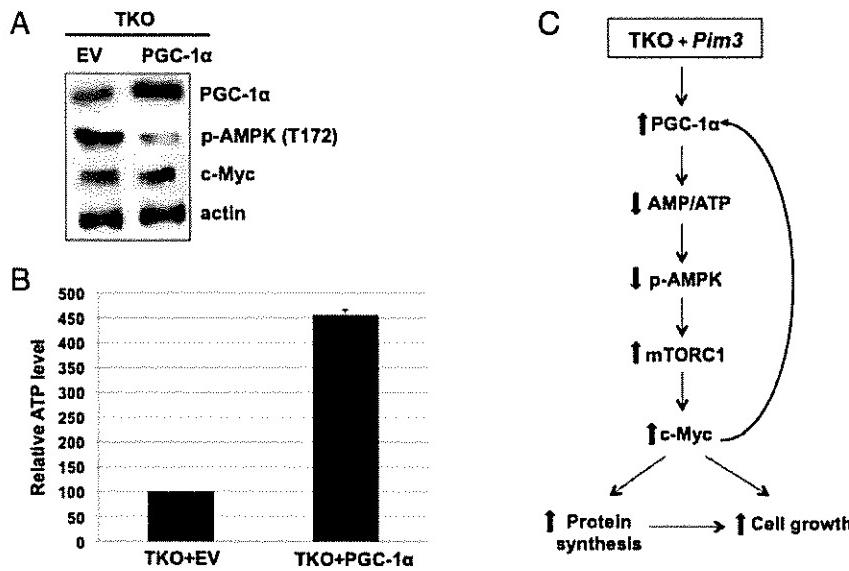
Fig. 5. Expression of PGC-1α restores the AMP:ATP ratio in TKO MEFs. (*A*) PGC-1α overexpression in TKO MEFs reduces AMPK activation. Lysates were prepared from TKO MEFs 48 h after transduction with empty vector (EV) or PGC-1α lentiviruses, and protein levels compared by Western blotting. (*B*) ATP levels determined in lysates from Fig. 5A as described in *Materials and Methods*. Values are the average of three independent measurements, and the standard deviation from the mean is shown. (*C*) Schematic summary of biologic changes observed in TKO MEFs expressing Pim-3.

AMPK and PGC-1α in the sensing and regulation of the cell's energy status, the levels of PGC-1α were investigated and found to be significantly lower in TKO MEFs. In comparison, Pim-3–containing MEFs showed increased levels of PGC-1α relative to WT. Therefore, in the case of the TKO MEFs, chronic AMPK activation coupled with drastically reduced levels of PGC-1α protein resulted in an elevated AMP:ATP ratio. Accordingly, infection of TKO MEFs with a lentivirus expressing PGC-1α was shown to increase ATP levels and decrease AMPK activation. The increased PGC-1α levels in Pim-3–only MEFs cannot be attributed solely to increased c-Myc because TKO/c-Myc MEFs showed lower levels of PGC-1α mRNA and protein relative to TKO/Pim-3 MEFs. This suggests the possibility that Pim-3 and c-Myc could cooperate in regulating PGC-1α levels in MEFs. This cooperation may extend beyond transcription/translation because PGC-1α levels and activity are regulated by multiple posttranslational mechanisms (37).

Pim-3 is the least-studied kinase of the Pim family; however, it has been linked to the development and progression of colon and pancreatic cancers (2–4, 44). Despite the high sequence identity and overlapping substrate specificity of the Pim kinases, Pim-3 expression alone is shown to overcome at least some of the defects found in the loss of both Pim-1 and Pim-2, including growth rate. Additionally, the knockout of Pim-1 and -2 and the expression of Pim-3 only led to a marked increase in c-Myc protein relative to WT MEFs. The observation that the transduction of Pim-1 or -2 into MEFs containing Pim-3 suppressed c-Myc levels suggested the possibility that individual Pim isoforms may regulate each other either directly or through substrate competition. This poses the question of whether Pim isoforms either individually or acting in concert regulate different biological processes and under what cellular circumstances. The question of the activity of Pim isoforms is of importance to the design of small-molecule inhibitors targeting these kinases and their use in the treatment of diseases, including cancer. Both Pim-1 and -2 are known to enhance c-Myc–induced transformation (6, 12) and phosphorylate and stabilize c-Myc protein, leading to increased transcriptional activity (9). In the MEFs used in this study, Pim-3 expression alone enhanced cap-dependent translation, increased c-Myc levels without changing the protein's stability, and increased the cell growth rate. Because elevated levels of both Pim-3 and c-Myc are found in

gastrointestinal cancers, our results suggest the possibility that Pim-3 might enhance the growth of these tumor cells in part by regulating c-Myc levels, thus highlighting the potential utility of Pim-3 targeted inhibitors.

Materials and Methods

Cell Culture. MEFs were derived from 14.5-d-old embryos and were genotyped as described (45). For stable cell lines, TKO MEFs were transduced with lentiviruses encoding empty vector, PIM-1, Pim-2, Pim-3, or c-Myc and selected with puromycin (4 µg/mL).

Construction of Lentiviral Vectors. The open reading frames of PIM-1 (human, 33 kDa isoform), PIM-2 (mouse), Pim-3 (mouse), c-Myc (mouse), and PGC-1α (human, a gift from Young-In Chi, Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY) were amplified by PCR from full-length cDNA clones and subcloned into the *AgeI-MluI* sites of pLex-MCS lentiviral vector (Open Biosystems). Methods for preparation of lentiviral stocks are detailed in *SI Materials and Methods*.

Quantitative RT-PCR (QT-PCR). Total RNA was isolated from MEFs using the RNeasy kit (Qiagen) according to the manufacturer's protocol. The first-strand cDNA was synthesized using Superscript first-strand synthesis kit and Oligo (dT) primer (Invitrogen).

Biochemical Analysis. K562 cells were transfected with scrambled siRNA or siPim-1 (ON-TARGETplus SMARTpool, Thermo Scientific) using Lipofectamine™2000 (Invitrogen) according to the manufacturer's protocol, and 48 h posttransfection lysates were prepared. Cell growth was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. ATP, ADP, and AMP were measured by HPLC as described previously (46), and ATP was also measured using the ATP Bioluminescence Assay Kit HS II (Roche) with 10^5 cells. eIF4E was captured on m^7-GTP sepharose (GE Lifesciences) from WT and TKO MEFs lysate and bound 4EBP1 and eIF4G determined by Western blotting.

^{35}S-Methionine Incorporation. Cells were serum starved for 1 h in methionine-free medium (Invitrogen), followed by labeling with 100 mCi of ^{35}S-methionine/mL. Lysates and labeled proteins were precipitated with trichloroacetic acid on glass microfiber filters (Whatman) using vacuum filtration, and ^{35}S-incorporation was counted.

Cap- vs. IRES-Dependent Translation. A bicistronic retroviral vector, pMSCV/rLuc-pol IRES-fLuc (a gift from Peter B. Bitterman, Department of Medicine, University of Minnesota, Minneapolis, MN), was used to produce viral particles for infecting WT, TKO and TKO/Pim-3 MEFs. Cells were collected

48 hr postinfection and Renilla/Firefly luciferase activities were quantified using the dual-luciferase reporter assay system (Promega) and a luminometer according to the manufacturer's instructions.

Polysome Profile Analysis. Sucrose density gradient centrifugation was employed to separate the ribosome fractions as described previously (47). c-Myc mRNA level in each fraction was measured by PCR.

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BIOCHEMISTRY

Therapeutics, Targets, and Chemical Biology

Cancer Research

Elevation of Receptor Tyrosine Kinases by Small Molecule AKT Inhibitors in Prostate Cancer Is Mediated by Pim-1

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Abstract

The PI3K/AKT pathway is hyperactivated in prostate cancer but its effective therapeutic targeting has proven difficult. In particular, the antitumor activity of AKT inhibitors is attenuated by upregulation of receptor tyrosine kinases (RTK) through an uncharacterized feedback mechanism. In this report, we show that RNA interference-mediated silencing or pharmacologic inhibition of Pim-1 activity curtails AKT inhibitor-induced upregulation of RTKs in prostate cancer cells. Although Pim kinases have been implicated in cap-dependent translational control, we find that in the context of AKT inhibition, the expression of RTKs is controlled by Pim-1 in a cap-independent manner by controlling internal ribosome entry. Combination of Pim and AKT inhibitors resulted in synergistic inhibition of prostate tumor growth *in vitro* and *in vivo*. Together, our results show that Pim-1 mediates resistance to AKT inhibition and suggest its targeting to improve the efficacy of AKT inhibitors in anticancer therapy. *Cancer Res; 73(11); 1–10. ©2013 AACR.*

Introduction

The PI3K/AKT pathway is commonly activated in human cancer and controls cellular processes that contribute to the initiation and maintenance of cancer (1). It is activated in 40% of primary and 70% of metastatic prostate cancers secondary to mutations or deletions in PTEN (1–3). Activation of the pathway can be associated with mutations in the phosphoinositide 3-kinase (PI3K) catalytic subunit P110 α and regulatory subunit (1), mutations in each of the 3 AKT isoforms (1, 4), and activation of receptor tyrosine kinases (RTK) by mutation (e.g., EGF receptor; EGFR) or gene amplification (e.g., HER2), which can result in activation of downstream PI3K/AKT (1, 5). Multiple small-molecule inhibitors have been developed to target PI3K/mTOR or AKT (6), but the efficacy of these drugs is compromised by the stimulation of compensatory signaling pathways that have the potential to enhance tumor growth (7–9). There is accumulating evidence that inhibition of the PI3K/AKT pathway can lead to adaptive resistance due to upregulation and activation of RTKs (7–9). The mechanism underlying the AKT inhibition-induced upregulation of some of these RTKs, including HER3, INSR, and insulin-like growth factor-1 receptor (IGF-IR), has been shown to, in part, involve FOXO transcription factors (7); however, these transcription factors do not seem to be involved in the AKT inhibition-induced upregulation of other RTKs, including MET, HER2, and RET (7).

The Pim family of serine/threonine kinases regulates cell survival pathways and has been implicated in the progression of several human cancers, including prostate cancer (10). Clinically, the expression of the Pim kinases is elevated in human prostate cancer (10), in which the PI3K/AKT pathway is activated, and the levels of Pim correlate with survival of patients with certain subtypes of human lymphoma (11), suggesting that the Pim kinases could play an important role in regulating tumor growth and, potentially, patient survival. As the Pim kinases have overlapping activity with AKT with both regulating apoptosis, cell-cycle progression, and cellular metabolism (12–13), and AKT and the Pim kinases share substrates in common (12–13), it has been suggested that Pim could play an important role in the activation of AKT (14). Reciprocal regulation of AKT and Pim-1 levels is suggested by the report that forced expression of nuclear-targeted AKT induces Pim-1 and either expression of a dominant-negative Pim-1 or genetic deletion of the enzyme increased AKT expression and phospho-AKT levels in cardiomyocytes (14).

Here, we show that inhibition of AKT leads to transcriptional induction of the Pim-1 protein kinase, and in turn, Pim-1 regulates the expression of RTKs. The anticancer activity of small-molecule AKT and Pim kinase inhibitors has been investigated.

Materials and Methods

Reagents and antibodies

GSK690693 was provided by GlaxoSmithKline for *in vitro* and *in vivo* studies. MK2206, PP242, AZD8055, and BEZ235 were purchased from Selleck Biochemicals. Antibodies are listed in the Supplementary Data.

Plasmids

The 5′-untranslated region (UTR) of human *Met* (15) was amplified by PCR using genomic DNA extracted from PC3-LN4

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cells as template with the following 2 primers: 5′-ATAC-TAGTGCTGCAGCGGCCGCGGTGGCTGA-3′ and 5′-AACCAT-GGCCCAACCTCCAGGATGTCGGCGCA-3′. The PCR product was sequenced and cloned into the EcoRI and NcoI sites of the plasmid of pRF to create pR-MET-F.

Immunoblotting

Cells were harvested in lysis buffer A consisting of 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% NP-40, and 5 mmol/L EDTA. Protein concentrations were determined by DC Protein Assay (Bio-Rad).

Cell culture and transfections

Cell lines were grown in RPMI (PC3-LN4, DU145, 22RV1, VCAP, and BT474) or Dulbecco's Modified Eagle Medium [HeLa, mouse embryo fibroblasts (MEF)] in 5% CO_2. DU145, 22RV1, VCAP, BT474, and HeLa cells were supplied by American Type Culture Collection and passaged in our laboratory for less than 6 months after receipt. PC3-LN4 cells were described before (16). The MEFs, which were triple knockout (TKO) for all Pim genes, were previously described (17). Cells were transfected with Lipofectamine 2000 reagent according to manufacturer's instructions.

Real-time PCR analyses

SYBR Green reactions were done using a BioRad iQ5 quantitative real-time PCR (qRT-PCR) system. For data analysis, raw counts were normalized to the housekeeping gene averaged for the same timepoint and condition (ΔC_t). Counts are reported as fold change relative to the untreated control ($2^{-\Delta\Delta Ct}$). All primers were designed and synthesized by Integrated DNA Technologies. Primers are listed in the Supplementary Data.

Luciferase assays

Firefly luciferase and *Renilla* luciferase activities were measured in a luminometer (Model TD 20/20; Turner Designs) using the reagents provided with the Dual Luciferase Reporter kit (Promega).

Soft-agar colony formation assays

The soft-agar assay was conducted on 6-well plates in duplicate. For each well, 5,000 cells were mixed in growth medium containing 0.7% agarose and GSK690693 or SMI-4a. Cells were then layered over 1% agarose in regular medium. Medium containing GSK690693 or SMI-4a was added to each well every 4 days. The assays were terminated after 21 days, and colonies were stained with crystal violet and counted under a microscope.

Cell proliferation measurement

Cells were plated in 96-well plates at 3,000 cells per well in 100 μL of 10% FBS-containing medium. After 24-hour incubation, the medium was replaced with 0.2% FBS medium with GSK690693, SMI-4a, or dimethyl sulfoxide (DMSO) for 72 hours. Cell viability was measured using a MTT assay. The absorbance was read at 590 nm with a reference filter of 620 nm.

In vitro transcription and RNA transfection

The mRNAs were purified with MEGA Clear Kit (Ambion), quantified spectrophotometrically, and their qualities were verified on a denaturing agarose gel. RNA transfection was conducted with *Trans*IT-mRNA Transfection Kit (Mirus) according to the manufacturer's suggestion. An aliquot of 1 μg of capped mRNAs and 2 μL of *Trans*IT-mRNA reagent together with 1 μL of mRNA boost reagent was used to transfect 80% confluent cells grown in 12-well plates. At 16 hours after transfection, cells were harvested and lysed for luciferase assay.

Animal experiments

Four- to six-week-old nu/nu nude male mice were obtained from Charles River Laboratories and maintained in pressurized ventilated caging. All studies were conducted in compliance with Institutional guidelines under an Institutional Animal Care and Use Committee-approved protocol (MUSC#3081). For efficacy studies, mice with well-established tumors were selected and randomized 14 days after implantation (size > 150 mm³); PC3-LN4 xenograft tumors were established in nude mice by subcutaneously injecting 5×10^6 cells suspended in PBS into the right flank. Mice were treated with vehicle, GSK690693, or SMI-4a, or GSK690693 + SMI-4a at the indicated doses. GSK690693 was dissolved in 30% propylene glycol, 5% Tween-80, 65% of 5% dextrose in water (pH4–5), and administered intraperitoneally daily, whereas SMI-4a was dissolved in the same solvent and administered by oral gavage twice daily. Tumor dimensions were measured with a caliper and tumor volumes were calculated [tumor volume (mm³) = (length × width²)/2].

Statistical analysis

The results of quantitative studies are reported as mean ± SD or mean ± SEM (for animal experiments). Differences were analyzed by Student t test. $P < 0.05$ was regarded as significant.

Results

AKT inhibition induces Pim-1 expression in prostate cancer cells

Treatment of the prostate cancer PC3-LN4 cells with the pan-AKT inhibitor GSK690693 markedly increased the levels of Pim-1 protein in a time- and concentration-dependent fashion (Fig. 1A and B) but had a minimal effect on the expression of Pim-3 protein and reduced the levels of Pim-2 (Fig. 1C). Similar results were obtained using another AKT inhibitor, MK2206, and a PI3K/mTOR dual inhibitor, BEZ235 (Fig. 1C). The induction of Pim-1 was also observed with GSK690693 treatment of human prostate cancer cell lines DU145, 22RV1, and VCAP (Supplementary Fig. S1A). The effect of GSK690693 on Pim-1 was not secondary to an off-target effect as knockdown in PC3-LN4 cells of all 3 AKTs with siRNAs increased the levels of Pim-1 protein (Fig. 1D). Treatment of PC3-LN4 cells with GSK690693 or MK2206 resulted in elevations in the level of Pim-1 mRNA, but not Pim-2 or Pim-3 (Fig. 1E). Similarly, treatment of PC3-LN4 cells with siRNAs directed at AKT1, AKT2, and AKT3 also resulted in the elevation of Pim-1 mRNA
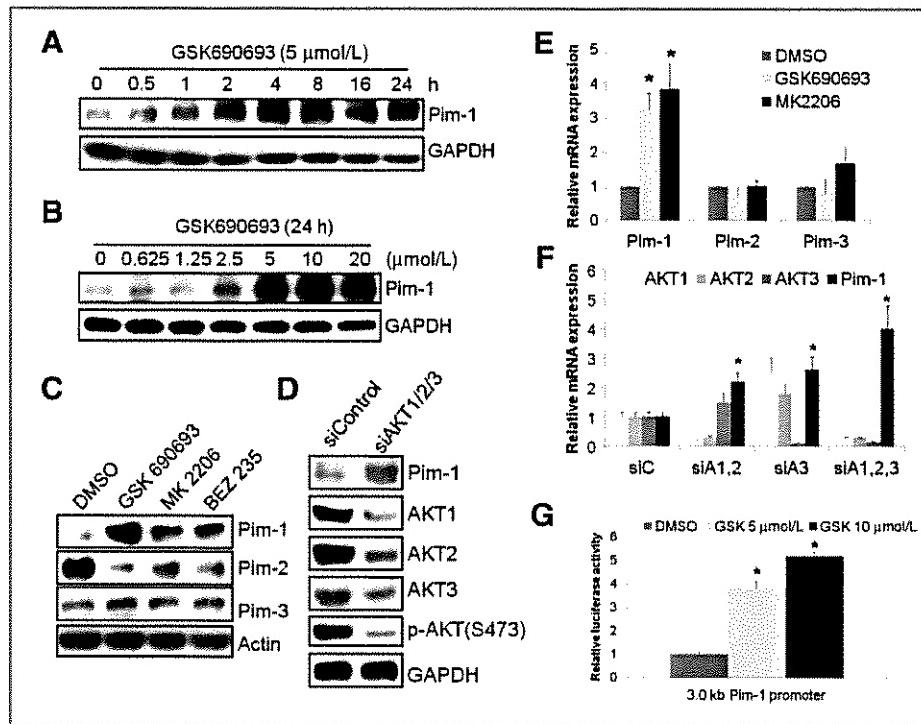
Figure 1. AKT inhibition induces expression of Pim-1. PC3-LN4 cells were treated with 5 μmol/L GSK690693 (A) for the times indicated, increasing doses of GSK690693 (B) as indicated for 24 hours, 5 μmol/L GSK690693, 2 μmol/L MK2206, or 0.5 μmol/L BEZ235 (C) for 24 hours, and siRNAs against AKT1, AKT2, and AKT3 or a negative control siRNA (D) for 72 hours. Whole-cell lysates were subjected to immunoblot analyses with the indicated antibodies. E, cells as in C were harvested and total RNA was isolated. Real-time qPCR analyses were conducted with Pim-1, Pim-2, Pim-3-specific primers. Results were normalized to the expression of β-actin. F, PC3-LN4 cells were treated with siRNAs against AKT1, 2 (siA1,2), AKT3 (siA3), AKT1, 2, 3 (siA1,2,3), or a nontargeting control siRNA (siC) for 72 hours and then RNA isolated, and real-time qPCR with indicated primers conducted. G, PC3-LN4 cells were transfected with a luciferase reporter containing a 3.0 kb human Pim-1 promoter. After 24 hours, cells were treated with DMSO or 2 different doses of GSK690693 (GSK) as indicated for additional 24 hours before harvesting for luciferase assays. Results were normalized to Renilla luciferase activity by a cotransfected plasmid carrying this enzyme. Data in E, F, and G are mean ± SD of 3 independent experiments. *, $P < 0.05$ compared with the corresponding negative control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Fig. 1F). To further determine whether GSK690693 regulates the transcription of the *Pim-1* gene, a 3.0 kb promoter fragment of the Pim-1 promoter was cloned upstream of a luciferase reporter. Addition of GSK690693 increased the activity of this promoter in PC3-LN4 cells (Fig. 1G).

Upregulation of Pim-1 is required for AKT inhibitor-associated induction of RTKs

Consistent with previous reports of upregulation of these RTKs in response to AKT inhibition (7), treatment of PC3-LN4 cells with GSK690693 increased the protein levels of multiple RTKs, including MET, EPHA2, RON, EGFR, HER2, HER3, INSR, and IGF-IR (Supplementary Fig. S1B). In addition, we observed increased extracellular signal-regulated kinase (ERK) phosphorylation resulting from treatment with GSK690693 (Supplementary Fig. S1B). This is in keeping with previous finding that PI3K inhibition leads to mitogen-activated protein kinase pathway activation (18–19).

GSK690693 also completely blocked the phosphorylation of 2 well-known AKT substrates, GSK3β and PRAS40, showing the effectiveness of this compound (Supplementary Fig. S1C) and caused the paradoxical hyperphosphorylation of AKT at its 2 regulatory sites (Thr308 and Ser473; Supplementary

Fig. S1C), a common property of ATP-competitive AKT inhibitors (20).

To determine whether Pim-1 plays an important regulatory role in the ability of AKT inhibitors to modulate RTKs, we first determined the effects of Pim-directed siRNAs and small-molecule inhibitors. The use of siRNA directed at Pim-1 showed that a forced reduction in Pim-1 levels markedly reduced the ability of GSK690693 to elevate the protein levels of multiple RTKs, including MET and EPHA2, HER3, HER2, INSR, and IGF-IR, as well as the phosphorylation of ERK (Fig. 2A). The addition of SMI-4a, a small-molecule Pim kinase inhibitor (21), reduced GSK690693-induced upregulation of RTK protein levels in PC3-LN4 (Fig. 2B), DU145, 22RV1, and VCAP cells (Supplementary Fig. S2)

The results of phospho-RTK antibody array (reverse-phase protein array; RPPA) analysis revealed that treatment of PC3-LN4 cells with GSK690693 increased the tyrosine phosphorylation of a number of RTKs tested in the assay, that is, MET, EPHA2, HER2, INSR, and EGFR (Supplementary Fig. S3). The lack of complete correlation in these assays may arise from the differing specificity of the antibodies used in the RPPA analysis. This change in RTK phosphorylation is consistent with the AKT inhibitor-induced increases in the protein levels of the
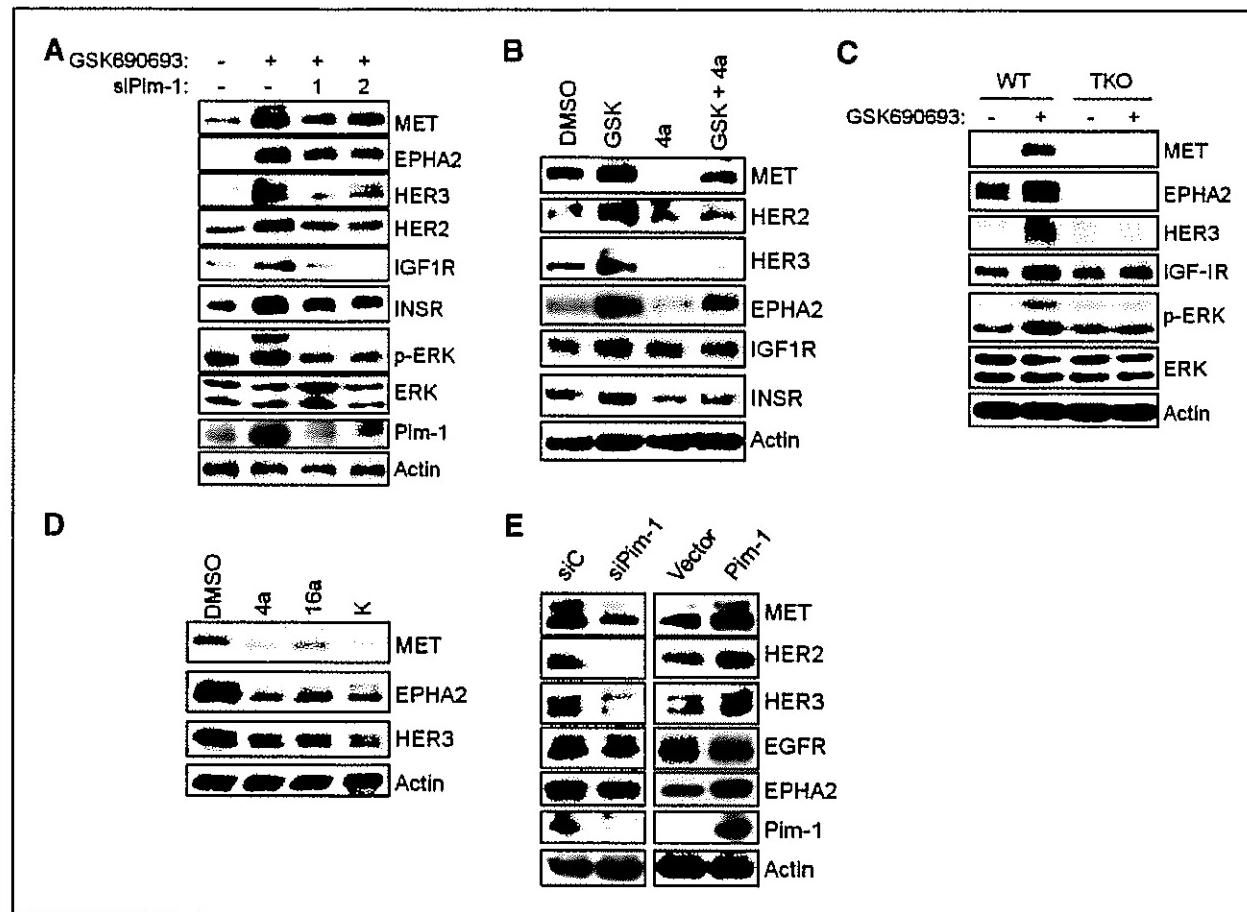
Figure 2. Pim-1 is required for elevated expression of RTKs induced by AKT inhibition. Immunoblot analyses were carried out with the indicated antibodies in PC3-LN4 cells that were treated with 2 different siRNAs (1 and 2) against Pim-1 as well as a nontargeting control siRNA (2 left lanes) for 48 hours followed by the addition of GSK690693 (5 μmol/L) for an additional 24 hours (A). B, PC3-LN4 cells were treated with GSK690693 (GSK, 5 μmol/L) or SMI-4a (4a, 10 μmol/L) or the combination of the 2 compounds for 24 hours. C–E, wild-type (WT; C), Pim kinase TKO murine embryonic fibroblast cells were treated with 5 μmol/L GSK690693 for 24 hours; PC3-LN4 cells were treated with 3 different Pim inhibitors, SMI-4a (4a, 10 μmol/L), SMI-16a (16a, 10 μmol/L), or K00135 (K, 5 μmol/L) for 24 hours (D); PC3-LN4 cells were transfected with a nontargeting control siRNA, siRNA against Pim-1, an empty vector, or a Pim-1-expressing plasmid for 72 hours (E).

RTKs; however, it cannot be ruled out that GSK690693 stimulates RTK phosphorylation through an alternative mechanism (7). Treatment with SMI-4a blocked the GSK690693-induced RTK phosphorylation (Supplementary Fig. S3), showing that the inhibition of Pim reverses the activity of this AKT inhibitor. To further evaluate the role of Pim-1 in regulating AKT inhibitor-induced upregulation of RTKs, MEFs were treated with GSK690693. In wild-type cells, but not in the Pim kinase-deficient (TKO) cells, GSK690693 treatment of the cells increased the levels of the RTKs tested, that is, MET, HER3, IGF-IR, and EPHA2 protein, as well as the phosphorylation of ERK (Fig. 2C). We treated PC3-LN4 (Fig. 2D) and VCAP (Supplementary Fig. S4) cells with 3 different Pim kinase inhibitors, SMI-4a, SMI-16a (21), and K00135 (22), to test whether Pim-1 activity affects the baseline level of RTK proteins in tumor cells. Treatment decreased the protein levels of the RTKs, for example, MET, EPHA2, and HER3, in both cell lines. Similarly, siRNA targeting of Pim-1 decreased the levels of

MET, HER3, HER2, and EGFR protein in PC3-LN4 cells (Fig. 2E). Conversely, overexpression of human Pim-1 in PC3-LN4 increased the levels of the RTKs, MET, HER3, EPHA2, HER2, and EGFR (Fig. 2E).

AKT inhibition increases cap-independent translation

AKT protein kinase activity controls protein synthesis by regulating the multistep process of mRNA translation at multiple stages from ribosome biogenesis to translation initiation and elongation (23). Although GSK690693 treatment of prostate cancer cells did not modify phosphorylation of 4E-BP1, this compound increased phosphorylation of eIF2α and eliminated phosphorylation of ribosomal protein S6 (Fig. 3A). To further define the role of cap-dependent translation in the mechanism of action of this agent, GSK690693 was combined with 2 potent inhibitors of mTOR complex (mTORC)-1/mTORC2 and thus cap-dependent translation, PP242 and AZD8055 (24–25). These inhibitors in combination with

Figure 3. AKT inhibition increases cap-independent translation. A, PC3-LN4 cells were treated with GSK690693 (5 µmol/L) alone or in combination with PP242 (2 µmol/L) or AZD8055 (1 µmol/L) for 24 hours and immunoblotting conducted. B, dicistronic luciferase plasmids containing viral (CrPV and HCV) or cellular (HIF1α, VEGF, and Myc) IRESs were transfected into PC3-LN4 cells. GSK690693 (5 µmol/L) was added 6 hours after transfection for additional 24 hours and luciferase activities were determined. Data are mean ± SD of 4 independent experiments. *, P < 0.05 compared with the corresponding DMSO control. C, PC3-LN4 cells were treated with increasing doses of GSK690693 as indicated for 24 hours and lysates examined by Western blotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
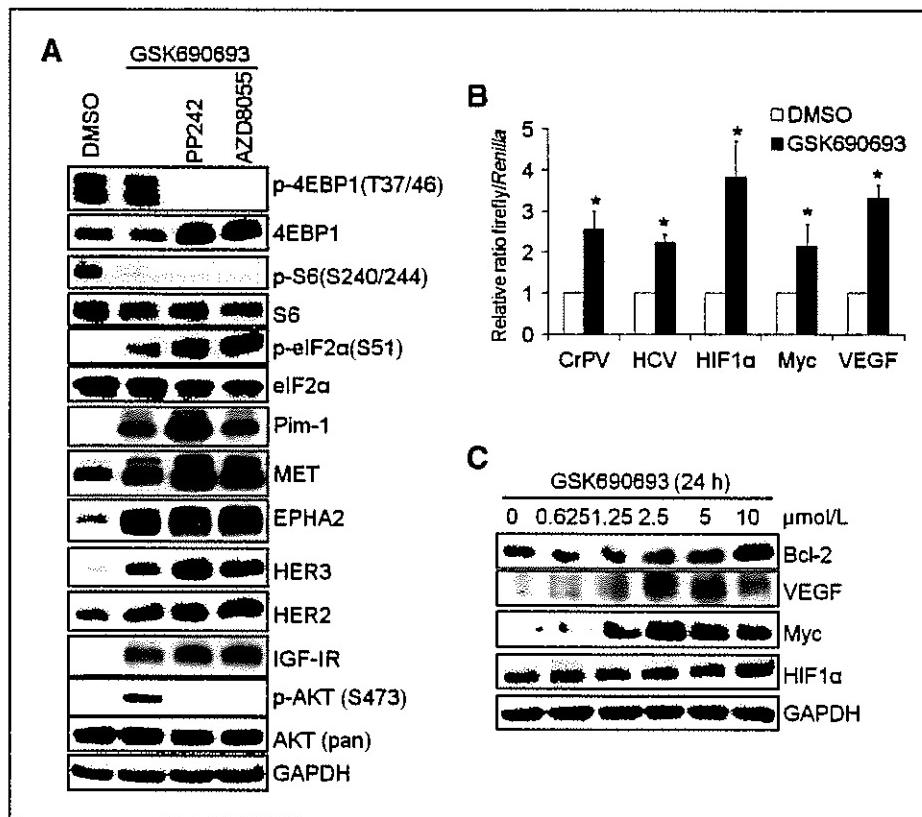
GSK690693 resulted in reduced phosphorylation of 4E-BP1 and increased eIF2α phosphorylation compared with GSK690693 alone (Fig. 3A), suggesting inhibition of 5'-cap-dependent translation. We measured the binding of eIF4G and 4E-BP1 to the 5' mRNA cap by using m⁷GTP-sepharose. The structure of these beads mimics the 5' mRNA cap and precipitates cap-interacting proteins. In agreement with the effect on phosphorylation of 4E-BP1, PP242, or AZD8055 in combination with GSK690693 strongly reduced eIF4G and increased 4E-BP1, binding to m⁷GTP-sepharose, whereas GSK690693 alone did not have a significant effect (Supplementary Fig. S5A). However, the treatment of prostate cancer cells with these mTORC1 inhibitors did not reduce the GSK690693-induced elevation of MET, EPHA2, HER3, and IGF-IR (Fig. 3A). A recent study (26) using Torin 1, an ATP-competitive mTOR inhibitor, showed that Torin 1-resistant mRNAs are enriched for RTKs such as MET, IGF-IR, and INSR, indicating that the translation initiation of these mRNAs do not depend on mTOR activity (27). We found that treatment of PC3-LN4 cells with PP242 or AZD8055 indeed did not inhibit the expression of MET, EPHA2, HER3, IGF1R, or INSR (Supplementary Fig. S5B). In addition, the expression of Bcl-2 whose translation under cellular stress (28) has been shown to be controlled by a cap-independent mechanism was not suppressed by treatment with mTOR inhibitors, whereas proteins known to be sensitive to mTOR inhibiton, YB-1, HSP90, RPS7 (26, 29), were reduced (Supplementary Fig. S5B). Reduced eIF4G and increased 4EBP1 binding to m⁷GTP-sepharose, and increased eIF2α phosphoryla-

tion (Supplementary Fig. S5C) confirmed that cap-dependent translation was efficiently inhibited. Together these data suggest that upregulation of RTKs is not controlled by cap-dependent mechanisms.

Under conditions of decreased cap-dependent translation, the internal ribosome entry site (IRES)-mediated translation can play a larger role in regulating protein synthesis (30). Recently, it has been shown that inhibition of PI3K/mTOR leads to increased IRES-mediated translation (8). Inhibition of AKT by GSK690693 resulted in increased IRES activity measured by ratio of firefly to Renilla luciferase activities in constructs containing either cellular [hypoxia-inducible factor-1α (HIF1α), Myc, and VEGF; ref. 31] or viral [cricket paralysis virus (CrPV) and hepatitis C virus (HCV); ref. 32] IRES sequences (Fig. 3B). In agreement with these findings, GSK690693 induced expression of Bcl-2, Myc, VEGF, and HIF1α, all of which can be translated in a cap-independent manner under cellular stress (28, 31, 33–34), further suggesting the possibility that cap-independent translation is upregulated (Fig. 3C).

Pim-1 regulates RTK expression through cap-independent translation

Expression of human Pim-1 in PC3-LN4 cells did not affect the levels of RTK mRNAs (Supplementary Fig. S6A and B) or the half life of the RTKs (Supplementary Fig. S6C), suggesting that Pim-1 may control the levels of these proteins through a translational mechanism. Plus, GSK690693 increased cap-

independent translation (Fig. 3). Taken together, we speculated that the upregulation of the RTKs induced by AKT inhibitors could be controlled, at least in part, by a cap-independent mechanism. We first determined whether the MET 5'-UTR contains an IRES that could be stimulated by either GSK690693 or Pim-1. The MET 5'-UTR is relatively long (408 nt) and is guanine-cytosine (GC)-rich (15), which are 2 common properties of IRES-containing 5'-UTRs. The 5'-UTR of MET was cloned and inserted in front of firefly luciferase in the dicistronic vector pRF (35). The presence of the MET 5'-UTR sequence increased the expression of downstream firefly luciferase relative to *Renilla* by 38-fold compared with the vector control (Fig. 4A), suggesting that it could function as an IRES. In comparison, the IRESs of encephalomyocarditis virus (EMCV), HIF1α, and VEGF produced 18-, 9-, and 13-fold increases, respectively. In PC3-LN4 cells transfected with the pRF vector containing the MET IRES, overexpression of Pim-1 or treatment of GSK690693 resulted in an increase in ratio of firefly to *Renilla* luciferase activities as compared with control treatment (Fig. 4A). Knockdown of Pim-1 suppressed GSK690603-induced MET IRES activities (Supplementary Fig. S7). Collectively, these results indicated that Pim-1 can potentially regulate translation of MET in a cap-independent fashion.

To determine whether the MET 5'-UTR is sufficient to drive translation by acting as an IRES and to rule out the possibility of a cryptic promoter in the 5'-UTR of MET, we *in vitro* transcribed the pRF vector containing the MET IRES yielding a capped dicistronic mRNA, and then transfected this mRNA directly into PC3-LN4 cells. Insertion of the MET or VEGF 5'-UTR resulted in a 7- or 5-fold increase in the firefly/*Renilla* ratio, respectively. In comparison, when the pRF vector containing the viral EMCV IRES was transcribed and transduced into these cells, the firefly/*Renilla* ratio increased by 114-fold (Fig. 4B). Thus, in comparison with a viral IRES, both the MET and VEGF sequences have relatively weak IRES activities. Besides MET, other RTKs including IGF-IR have been reported to have IRES elements in their 5'-UTRs (36). As shown in Fig. 4C, the IRES activity of the 5'-UTR of IGF-IR was increased on treatment of the cells with GSK690693 or Pim-1 overexpression and, conversely, was decreased on knockdown of endogenous Pim-1 protein levels. Furthermore, knockdown of Pim-1 suppressed GSK690603-induced IGF-IR IRES activities (Supplementary Fig. S7). It is possible that this mechanism is important for the control of other RTKs because in general these genes have long 5'-UTRs. In addition, knockdown of Pim-1 in PC3-LN4 cells led to a reduction of IRES activities of viral, CrPV
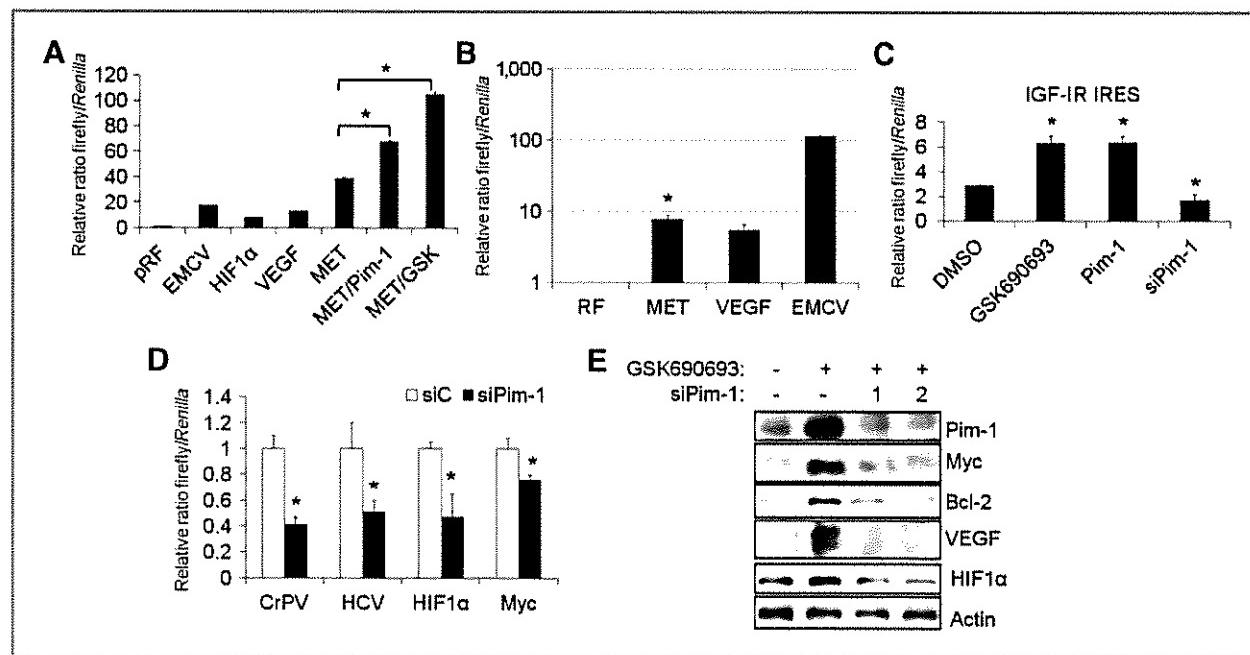

Figure 4. Pim-1 regulates RTK translation by controlling IRES activity. A, dicistronic plasmids pRF, pR-EMCV-F, pR-HIF-F, pR-VEGF-F, and pR-MET-F were transfected into PC3-LN4 cells. A Pim-1–expressing plasmid was cotransfected with pR-MET-F as indicated. GSK690693 (GSK, 5 µmol/L) was added 6 hours after transfection and luciferase activities were determined 24 hours after transfection. Data are mean ± SD of 4 independent experiments. *, $P < 0.05$ compared with the MET. B, capped, polyadenylated dicistronic mRNAs were transfected into PC3-LN4 cells. The ratios of firefly/*Renilla* activities are shown relative to the ratio for RF, which was given a value of 1. C, a dicistronic plasmid containing IGF-IR IRES was transfected into PC3-LN4 cells with or without either a Pim-1–expressing plasmid or siRNA targeted at Pim-1. GSK690693 (GSK, 5 µmol/L) was added 6 hours after transfection and at 48 hours luciferase activities were determined. D, dicistronic luciferase plasmids containing viral (CrPV and HCV) or cellular (HIF1α and Myc) IRESs were transfected into PC3-LN4 cells together with siRNA against Pim-1 or a nontargeting control siRNA, and luciferase activities were determined 48 hours after transfection. E, PC3-LN4 cells were treated with 2 different siRNAs (1 and 2) against Pim-1 as well as a nontargeting control siRNA (2 left lanes) for 48 hours followed by adding GSK690693 (5 µmol/L) for additional 24 hours as indicated. Whole-cell lysates were subjected to immunoblot analyses with the indicated antibodies. Data in B, C, D, and E reflect the mean ± SD of 4 independent experiments. *, $P < 0.05$ compared with corresponding negative controls.

and HCV, and cellular, HIF1α and Myc, IRESs (Fig. 4D). These data suggest that Pim-1 could be a more general regulator of IRES-mediated translation. This concept is further supported by our finding that the upregulation of proteins whose translation can be controlled by an IRES-mediated mechanism under cellular stress, Bcl-2, Myc, VEGF, and HIF1α, is stimulated by GSK690693 and requires Pim-1 expression (Fig. 4E).

Ribosomal stress abrogates AKT inhibition-induced upregulation of RTK expression

Pim-1 has been shown to physically interact with ribosomal protein S19 and to cosediment with ribosomes (37–38). Knockdown of ribosomal protein S19 or S6 abolished upregulation of MET, EPHA2, HER3, and IGF-IR induced by GSK690693 without affecting Pim-1 induction (Fig. 5A). Consistent with findings from other laboratories (38–40), reduced protein expression of ribosomal protein S6 was seen when S19 was decreased by siRNA and vice versa (Fig. 5A). To test the effect of ribosomal stress on RTK upregulation independent of ribosomal protein knockdowns, low concentrations of actinomycin D (ActD) were used to inhibit RNA polymerase I, and thus induce ribosomal stress (41–42). Similar to S19 and S6 knockdowns, ActD treatment blocked upregulation of MET, EPHA2, HER3, and IGF-IR induced by GSK690693 (Fig. 5B). ActD treatment also inhibited upregulation of MET, EPHA2, and HER3 resulting from direct Pim-1 overexpression in PC3-LN4 cells (Fig. 5C). Ribosomal stress did not seem to affect global translation as the expression of Src and ERK1/2 proteins was not altered (Fig. 5A and B). These data suggest that Pim-1 may work through intact ribosomes to control RTK expression.

Combination treatment with an AKT and a Pim inhibitor synergistically blocks prostate tumor growth in vitro and in vivo

As a preliminary test of whether combined inhibition of AKT and Pim kinases might provide synergistic antitumor efficacy, we tested the effects of the inhibitors on the proliferation of PC3-LN4 cells in vitro. Treatment of PC3-LN4 cells with the Pim inhibitor SMI-4a in combination with the AKT inhibitor GSK690693 resulted in a synergistic enhancement of the inhibition of proliferation as shown by combination index of less than 0.5 (Fig. 6A; data not shown), and a markedly greater reduction in both the numbers and the size of colonies seen in a soft-agar colony formation assay (Fig. 6B). GSK690693 and SMI-4a blocked the proliferation of DU145 in a similar fashion (Supplementary Fig. S8).

To test the activity of these agents in vivo, PC3-LN4 cells were injected into mice and treated with GSK690693 alone, SMI-4a alone, or both drugs in combination on a daily basis for 21 days starting at 15 days after tumor implantation. When used alone, treatment of these drugs caused a modest inhibition of tumor growth, whereas the combined treatment resulted in a markedly greater inhibition of tumor growth (Fig. 6C). As shown in Fig. 6D, immunoblot analysis of lysates of tumors harvested at the termination of the experiment on day 36 had upregulated the levels of MET, EPHA2, and HER3 protein in mice treated with GSK690693 as compared with the tumors from mice treated with vehicle (Fig. 6D). Interestingly, the levels of Pim-1 were increased in the combined therapy, and could suggest an in vivo interaction between these agents cannot be ruled out. This upregulation of the RTKs was significantly reduced in the tumors from mice treated with a combination of GSK690693 and SMI-4a (Fig. 6D).
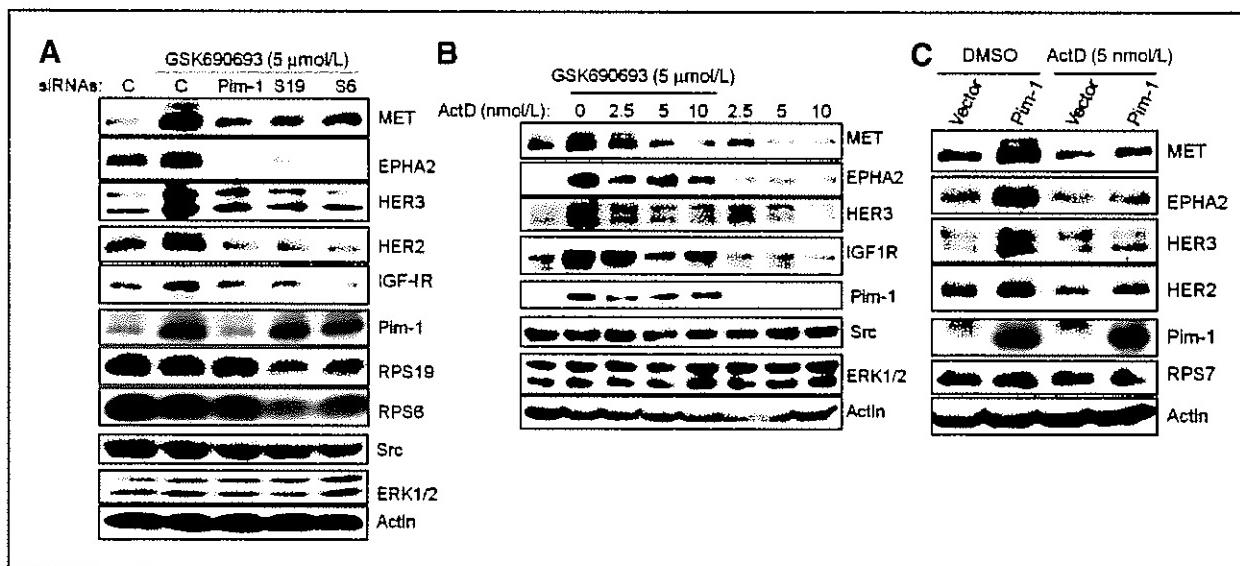

Figure 5. Ribosomal stress abrogates RTK upregulation induced by GSK690693. A, PC3-LN4 cells were treated for 48 hours with siRNAs against Pim-1, ribosomal protein S19, S6 as well as a nontargeting control siRNA (2 left lanes) followed by adding GSK690693 (5 μmol/L) for an additional 24 hours. B, PC3-LN4 cells were treated with increasing dose of ActD with and without 5 μmol/L GSK690693 for 24 hours. C, PC3-LN4 cells were transfected with a Pim-1–expressing plasmid or a control vector. ActD (5 nmol/L) was added 24 hours after transfection for an additional 16 hours. Whole-cell lysates were subjected to immunoblot analyses with the indicated antibodies.
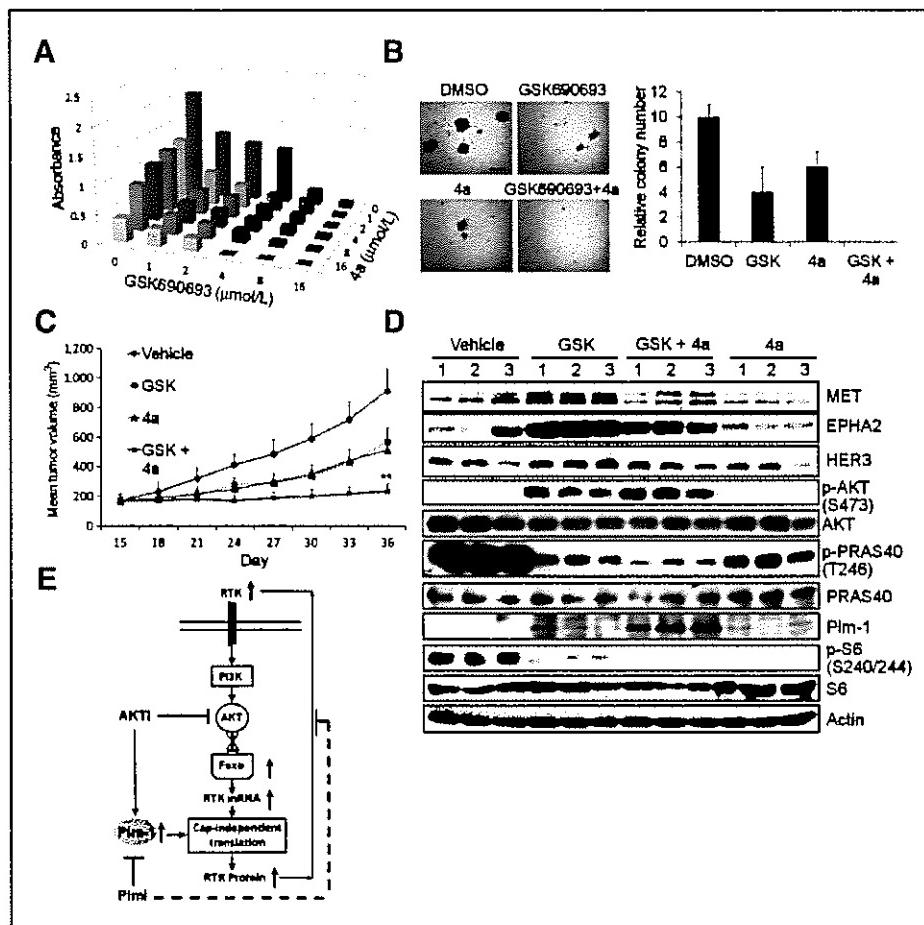
Figure 6. Combined inhibition of AKT and Pim shows synergistic antitumor activity. A, PC3-LN4 cells were treated with increasing doses of GSK690693 and SMI-4a (4a) as indicated in media containing 0.2% serum for 72 hours followed by a MTT assay. Similar results were obtained from 3 independent experiments. One representative experiment is shown. B, PC3-LN4 cells were plated in 10% serum and 0.7% agarose-containing medium with 10 μmol/L of GSK690693 or SMI-4a alone or in combination. Colonies were stained with crystal violet and counted after 21 days and the data are mean ± SD of 3 independent experiments. Bar, 200 micrometer. C, Nu/Nu mice bearing PC3-LN4 tumors were randomized into 4 groups: vehicle, GSK690693 (30 mg/kg i.p. daily), SMI-4a (60 mg/kg oral twice/day), and the combination. Tumor size was measured every 3 days. The results are presented as the mean tumor volume ± SEM ($n = 6$ mice/group). **, $P < 0.02$ for the GSK + SMI-4a group versus all other treatment groups. D, immunoblot analyses of tumors in C with the indicated antibodies. Tumors were harvested on day 36, 6 hours after the last dose of therapy. The numbers above each lane represent individual tumors in that treatment group. E, a model for the feedback upregulation of RTK expression mediated by Pim-1 kinase.

Discussion

The results of these experiments provide insights into the mechanisms underlying the compensatory interplay between AKT and Pim-1 in the regulation of prostate cancer cell behavior influenced by the expression of RTKs. They suggest a model in which reduction in AKT activity is associated with an increase in the levels of Pim-1 protein kinase that occurs through a transcriptional mechanism. This increase in Pim-1 kinase is associated, in turn, with promotion of the expression of RTKs through a cap-independent mechanism. Downregulation of Pim-1 blocks the feedback elevation in RTKs associated with inhibition of AKT (Fig. 6E). Likewise inhibitors of Pim synergize with small-molecule AKT inhibitors to block the growth of prostate cancer cells.

The control of Pim-1 protein levels is complex and has been shown to involve the ubiquitin proteasome pathway and

translational mechanisms (43). In the current study, we show that inhibition of AKT can increase the levels of Pim-1 through a transcriptional mechanism; however, it is possible that additional alternative mechanisms could also play a role in increasing Pim-1 protein levels. The induction of Pim-1 by AKT inhibition coincides with suppression of total protein synthesis (Supplementary Fig. S9) and is not inhibited by further treatment with mTORC inhibitors (Fig. 3A), suggesting that Pim-1 protein levels could also be regulated in a cap-independent manner. The Pim-1 5'-UTR may contain an IRES that could also be regulated by specific cellular growth conditions (44), although the existence of this IRES is controversial (45).

It has been shown previously that inhibition of AKT regulates the transcription of RTKs by modulating the activity of Foxo transcription factors (7); however, in the same study no change was seen in the level of HER2, RET, or MET mRNAs,

suggesting that the levels of specific RTKs might be controlled by other mechanisms. Cap-dependent translation plays a role in both PI3K/AKT and Pim-2 enhancement of the synthesis of specific proteins (46). It should be noted, however, that molecules that blocked mTORC1 activity could not inhibit the Pim-2 protein kinase and an agent that blocked eIF4A function, which is known to take part in IRES mediated translation, was required. In addition, small-molecule mTORC inhibitors can decrease the translation of many mRNAs, for example, 5'-terminal oligopyrimidine tracts mRNAs, while increasing the level of translation of RTKs (26), again suggesting that these RTK mRNAs may be translated in a cap-independent fashion. Moreover, further inhibition of cap-dependent translation with the mTORC1/2 inhibitors, PP242 and AZD8055, had no effect on the ability of GSK690693 or Pim-1 to induce RTKs (Fig. 3A), suggesting that in the experimental conditions used in these studies, the mechanism by which this agent controls RTK levels is not cap dependent.

Our results are consistent with the hypothesis put forward by Muranen and colleagues that inhibition of PI3K/mTOR could lead to enhanced cap-independent translation (8). Cloning of the Met 5'-UTR into a dicistronic luciferase vector showed that it can function as an IRES element, although weakly in comparison with viral sequences, and its activity is enhanced by GSK690693 and Pim-1 overexpression (Fig. 4A and B). Further supporting evidence of the ability of GSK690693 and Pim-1 to regulate the activity of the IRES is the observation that the IGF-IR IRES (47) is stimulated by these agents and that Pim-1 knockdown decreases the activity of this element (Fig. 4C). Our data further suggest that Pim-1 may be essential for full IRES activity of additional viral and cellular IRES elements, including HCV, CrPV, HIF1α, and Myc (Fig. 4D), suggesting a general role of Pim in the control of cap-independent translation.

It has been suggested previously that because they are both survival kinases, AKT and Pim protein kinases could be important pharmacologic targets to inhibit tumor growth (12). Our experiments show a high degree of synergism between small-molecule inhibitors of AKT and Pim in their ability to kill prostate cancer cells both in tissue culture and in a xenograft model (Fig. 6). Analysis of the tumors from treated animals showed that AKT inhibitor treatment elevates RTKs in the tumor cells grown *in vivo* and that simultaneous treatment with a Pim inhibitor downregulates this effect (Fig. 6D). Because both these kinase pathways are highly activated in human prostate cancer, dual inhibitor treatment of these tumors could be a particularly attractive chemotherapeutic strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by the authors.

Authors' Contributions

Conception and design: B. Cen, A.S. Kraft
Development of methodology: B. Cen, W. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Cen, W. Wang, A.S. Kraft
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Cen, S. Mahajan, A.S. Kraft
Writing, review, and/or revision of the manuscript: B. Cen, A.S. Kraft
Study supervision: B. Cen, A.S. Kraft

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